EFFECT OF COUROUPTIA GUIANENSIS ON N-DIETHYLNITROSAMINE INDUCED OXIDATIVE STRESS IN WISTAR RATS

Dissertation Submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY

Chennai-32

In Partial fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

SUBMITTED BY

Reg.No. 26103097

Under the guidance of

Mr. V. RAJESH., M. PHARM

DEPARTMENT OF PHARMACOLOGY

J.K.K.NATTRAJA COLLEGE OF PHARMACY

KOMARAPALAYAM - 638 183,TAMILNADU.

MAY-2012

Certificate

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Effect of courouptia guianensis on N-diethylnitrosamine induced oxidative stres in wistar rats"** submitted by the student bearing **Reg. No. 26103097** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **MASTER OF PHARMACY** in **PHARMACOLOGY** was evaluated by us during the examination held on…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation **"Effect of courouptia guianensis on N-diethylnitrosamine induced oxidative stres in wistar rats",** submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, was carried out by **Ms. K.V. KAVITHA [Reg.No. 26103097],** in the Partial fulfillment of award of degree of **MASTER OF PHARMACY** in Pharmacology under direct supervision of **Mr. V. RAJESH, M. Pharm,** H.O.D of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam, during the academic year 2011-2012.

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सेवा में / To

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महोदया/Madam,

The plant specimen brought by you for identification is identified as *Couroupita guianensis* Aublet - LECYTHIDACEAE

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

GVSMurthy 19/9/11

(डॉ. जी.वी.एस. मूर्ति /Dr. G.V.S. Murthy)
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Committee for the purpose of control and supervision of experiments on animals (CPCSEA),

Institutional Animal Ethics Committee (IAEC).

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CERTIFICATE

Title of the Project	:	Effect of *Courouptia guianensis* flowers on N-diethyl nitrosamine induced oxidative stress on Wistar rats.
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Name of IAEC Chairperson	:	**Dr. P. Perumal**

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DECLARATION

The work presented in this dissertation entitled **"Effect of courouptia guianensis on N-diethylnitrosamine induced oxidative stres in wistar rats",** was carried out by me, under the direct supervision **V.RAJESH, M. Pharm,** H.O.D of pharmacology., J.K.K. Nattraja College of Pharmacy, Komarapalayam.

I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university and the thesis is ready for evaluation.

PLACE : Komarapalayam **K.V.KAVITHA,**

DATE : **Reg.No: 26103097.**

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K.V.KAVITHA

Dedicated to

Almighty

My Beloved Parents

And

My Husband

Contents

CONTENTS

LIST OF ABBREVIATIONS USED

LPO	-	Lipid peroxidation
MDA	-	Melon di aldehyde
Conc	-	Concentrated
Hb	-	Hemoglobin
GI	-	Gastro Intestinal
ALP	-	Alkaline Phosphatase
TB	-	Total Bilirubin
DNA	-	Deoxy ribo nucleic acid
TNF	-	Tumor Necrosis Factor
IF	-	Interferons
IL	-	Interleukins
RNA	-	Riboxy nucleic acid
Ccl_4	-	Carbon tetrachloride
INH	-	Isoniazid
ACH_Z	-	Acetyl hydrazine
GSH	-	Glutathione reductase
H_2O_2	-	Hydrogen peroxide
LFTs	-	Liver Function Tests
ALT	-	Alkaline transaminase

AST	-	Aspartate amino transferase
FBS	-	Fasting Blood glucose
TG	-	Triglyceride
TC	-	Total Cholesterol
LDL	-	Low Density Lipoprotein
VLDL	-	Very Low Density Lipoprotein
HDL	-	High Density Lipoprotein
AI	-	Atherogenic
CA	-	Coronary artery
WBC	-	White Blood Cells
HCL	-	Hydrochloric acid
CPCSEA	-	Committee for the purpose of control and supervision on experimental animals
CMC	-	Carboxy Methyl Cellulose
µl	-	Micro litre
Wt	-	Weight
%w/w	-	Percent weight per weight
% v/v	-	Percent volume per volume
GP_x	-	Glutathione peroxidase
SOD	-	Superoxide dismutase
OECD	-	Organization for Economic Co-operation and Development

IU/L	-	International Units per Litre
g/dl	-	gram per deci litre
mg/dl	-	milli gram per deci litre
gms	-	grams
mg/kg	-	milli gram per kilo gram
nmol	-	nano mole
U/mg	-	Units per milli gram
%	-	Percentage
Kg	-	Kilogram
IP	-	Intra Peritoneal
SC	-	Subcutaneous
LCAT	-	Lecithin cholesterol acyl transferase
LDL-c	-	low density lipoprotein-cholesterol
HDL-c	-	High density lipoprotein-cholesterol
LPL	-	Lipoprotein lipase
LRP	-	LDL-receptor related protein
GPO	-	Glycerol-3-phosphate oxidase
EDTA	-	Ethylene Diamine Tetra Acetic acid
CVD	-	Cardio Vascular Disease
CAD	-	Coronary Artery Disease
CAT	-	Catalase

Chapter I

Introduction

INTRODUCTION

INTRODUCTION TO HERBAL MEDICINE

Nature is enriched with pharmacologically active molecules which have been used for the treatment of various incurable diseases **(kokate et al., 2000; Ravi et al., 2009; Trease and Evans, 1983)**. Herbal medicines are recommended for different kind of biological activity for health care needs **(Najiah et al., 2011; Nithya and Baskar, 2011)**. The basic source of knowledge of modern medicine is plants. Herbal medicine also called herbology or botanical medicine. Products obtained from plant source are used for the treatment in wide variety of forms without any chemical modification.

About 75 to 80% of world population is using herbal medicine for primary health care because of less side effects, good compatability with human body and good cultural acceptability **(Karim et al., 2011; Premanath et al.,2011; Kapoor and Saraf, 2011)**. The trend of using herbal medicines has increased enormously. Herbal medicines, derived from scientific heritage and ancient civilization. Renewable sources of raw materials are used for making herbal drugs by eco friendly process and they are used for certain diseases where no modern medicine is available. All parts of plants contain various medicinal properties (**Mukherjee et al 2002**). The plant extracts and its active constituents are screened for various pharmacological activities. Herbalism has a long tradition of use and it contains wide variety of chemical compounds used to treat many diseases.

Auyrvedha is a holistic traditional health care system in which human body, mind and soul are taken into consideration for treatment. There is a world wide belief that herbal medicines are safer and less damaging to human body than modern medicines (**Kraft et al 2007**). The use of herbal medicine is enormously increased as science began to take a closer look at herbal remedies. As malnutrition and poverty is overruling in India, plant derived products will reduce the cost of health care. So herbal medicines are widely acceptable among people and India has a rich history of using herbal medicines for various treatments. India is enriched with variety of flora due to different climatic conditions. Among 500,000 species of

plants on earth, about 5000 of them have been studied by modern science for medicinal purpose.

Herbal formulations now serve as a basis of drug discovery initially it was dispensed in the form of crude drugs such as tinctures, powders, teas, juices and other formulations. All plant parts and its extracts have been used in herbal medicine over the centuries. The world health organization recently defined traditional medicine including herbal drugs that, it is a synthesis of generations of therapeutic experience of practicing physicians of indigenous system of medicine. Traditional preparations include medicinal plants, organic matter and minerals where herbal drugs constitute medicinal plants primarly used for health therapy. Recently modern medicines are derived from plant source with some modification to improve the activity. The parts of medicinal plants should be standardized on the basis of their major compounds and subjected to limited safety studies in animal before marketing.

History of herbal medicine

Herbal medicine is an oldest form of health care system used by all cultures throughout history. In 20^{th} century much of the scientific medicine was derived from herbal lore of native peoples. Researchers found that people in different parts of the world used herbs as medicine for their health care. In the early 19^{th} century, active ingredients from the plants were extracted and modified by the scientist and later chemists made their own version of plant compounds.

The first written herbal record was in 2800 BC in china and western herbal medicine dated back to ancient Greece. Hippocrates wrote first herbal medicine in Greek. A classification system of herbal remedies and illness was developed by an herbal practitioner, Galen in 200 AD. Europeans used herbs as medicine in 15^{th} century. Chinese emperor Shen Nong wrote an authoritative treatise on herbs and that is using still today. In 1941, pharmacist and medicine act is passed which gave rights to pharmacists to dispense herbal medicines. The british herbal medicine association was also formed and published british herbal pharmacopeia (**www.herbal/supplement/resource.com**).

Recently World Health Organization estimated that more than 80% of world population is using herbal medicine to treat diseases. In 1989 World health assembly adopted a resolution about the importance of herbal medicines in individuals and communities. WHO developed guidelines for herbal medicine assessment and it was ratified by 6th international conference of Drug and Regulatory Authorities held at Ottawa in same year. WHO guidelines include quality assessment, safety assessment, stability and toxicology studies. All scientific generated data projected herbal medicine in a proper perspective and sustained in a global market.

Evidence and importance scientific of herbal medicine

Several clinical trails are done on herbs in recent years, many of them have shown that herb is a safe and effective alternative to modern medicines. One recent study compared the quality of clinical trails of phytomedicine to matched trails with modern medicines and concluded that method and reporting quality of western clinical trails of phytomedicines was on superior to modern medicine(**Nartley et al 2007**). While evaluating any clinical study, it is important to consider quality and design of the study and factors include nature of medicine investigated, goal of the study and how they were measured, dose, length of the treatment. Many pharmaceutical companies are now conducting researches on plant material which is collected from rain forest and other places for therapeutic purpose (**Spinella et al 2002**).

Treatment with herbal medicine is holistic. The approach involves “balancing the body’s vital energy” with a belief that it can treat any diseases. Almost 25% of conventional medicines are based on plant origins, example: aspirin, quinine, digitalis etc. Pharmacologist, botanist and microbiologist are searching new herbal medicines in different parts of the world for health care needs. Drug discovery from plants begins with botanists, ethnobotanist, ethnopharmacologists or plant ecologists who will collect and identifies the plant. Molecular biology plays an important role in medicinal plant drug discovery for determination of appropriate screening assays towards relevent molecular targets. Herbal medicines must be prepared according to good manufacturing practices. The specific active constituents in a herb works to treat diseases. The identification of active principles and

evaluation of extracts should ensure safety and effective pharmacological activity (**Prakash et al 1998**).

India has well recorded and well practiced knowledge of traditional herbal medicine. The regulatory agency should take a preventive measure against the misuse of herbal medicines as was done by US-FDA by banning dietary supplement cholestin. Recently drugs are applied to standardization procedures to elucidate analytical marker compounds. For the entry of herbal medicine into the developed countries, the basic requirements include well documented traditional use, single plant medicines, safety, stability, standardization based on activity, plant medicines should be free from pesticides, heavy metals and pharmacological activity studies in animals. All these data are the supportive measures for the herbal medicine and it has gained much more importance in the field of medicine (**Kamboj et al 2000**).

Oxidative stress

Oxidative stress is a general term which is used to describe the steady state level of oxidative damage in a cell, tissue or organ caused by the reactive oxygen species. This damage can affect a entire organism or a specific molecule. It is a imbalance between the generations of oxygen derived radicals and the organism's antioxidant potential (**devasagayam et al 1995**). Through the production of peroxides and free radicals, toxic effects are produced as a result of disturbances in the normal redox state of tissues that damage all components of the cell, including proteins, lipids and DNA. Reactive oxygen species and oxidative stress in liver cells plays an important role in liver diseases. Some reactive oxidative species can even act as a messangers through a redox signaling phenomenon.

Free Radicals and Reactive Oxygen

A radical (often, but unnecessarily called a free radical) is an atom or group of atoms that have one or more unpaired electrons. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can wreak havoc on a broad range of macromolecules. A

prominent feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells.

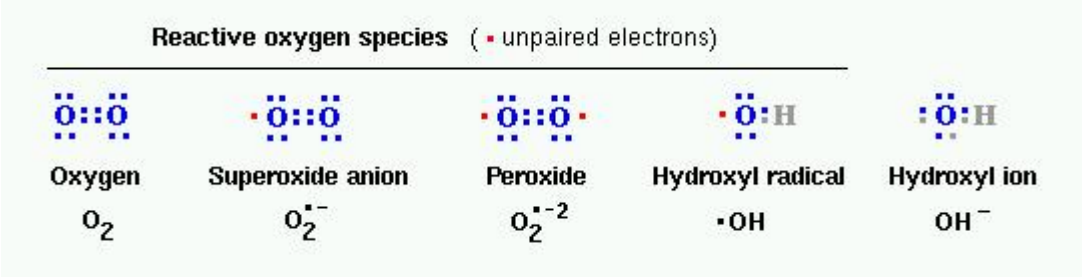
Oxygen Radicals

There are many types of radicals, but those of most concern in biological systems are derived from oxygen, and known collectively as reactive oxygen species. Oxygen has two unpaired electrons in seperate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation.

Sequential reduction of molecular oxygen (equivalent to sequential addition of electrons) leads to formation of a group of reactive oxygen species:

- **superoxide anion**
- **peroxide** (hydrogen peroxide)
- **hydroxyl radical**

The structure of these radicals is shown in the figure below, along with the notation used to denote them. Note the difference between hydroxyl radical and hydroxyl ion, which is not a radical.



Another radical derived from oxygen is **singlet oxygen**, designated as $^1O_2$. This is an excited form of oxygen in which one of the electrons jumps to a superior orbital following absorption of energy.

Formation of Reactive Oxygen Species

Oxygen-derived radicals are generated constantly as part of normal aerobic life. They are formed in mitochondria as oxygen is reduced along the electron transport chain. Reactive oxygen species are also formed as necessary intermediates in a variety of enzyme reactions. Examples of situations in which oxygen radicals are overproduced in cells include:

- **White blood cells** such as neutrophils specialize in producing oxygen radicals, which are used in host defense to kill invading pathogens.

- **Cells exposed to abnormal environments** such as hypoxia or hyperoxia generate abundant and often damaging reactive oxygen species. A number of drugs have oxidizing effects on cells and lead to production of oxygen radicals.

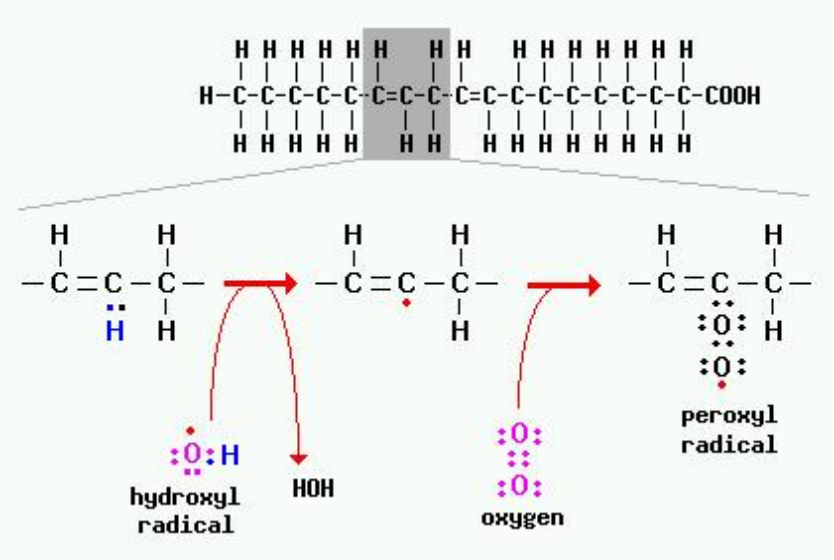
- **Ionizing radiation** is well known to generate oxygen radicals within biological systems. Interestingly, the damaging effects of radiation are higher in well oxygenated tissues than in tissues deficient in oxygen.

Biological Effects of Reactive Oxygen

It is best not to think of oxygen radicals as "bad". They are generated in a number of reactions essential to life and, as mentioned above, phagocytic cells generate radicals to kill invading pathogens. There is also a large body evidence indicating that oxygen radicals are involved in intercellular and intracellular signalling. For example, addition of superoxide or hydrogen peroxide to a variety of cultured cells leads to an increased rate of DNA replication and cell proliferation - in other words, these radicals function as mitogens.

Despite their beneficial activities, reactive oxygen species clearly can be toxic to cells. By definition, radicals possess an unpaired electron, which makes them highly reactive and thereby able to damage all macromolecules, including lipids, proteins and nucleic acids.

One of the best known toxic effects of oxygen radicals is damage to cellular membranes (plasma, mitochondrial and endomembrane systems), which is initiated by a process known as lipid peroxidation. A common target for peroxidation is unsaturated fatty acids present in membrane phospholipids. A peroxidation reaction involving a fatty acid is depicted in the figure below.



Reactions involving radicals occur in chain reactions. Note in the figure above that a hydrogen is abstracted from the fatty acid by hydroxyl radical, leaving a carbon-centered radical as part of the fatty acid. That radical then reacts with oxygen to yield the peroxy radical, which can then react with other fatty acids or proteins.

Peroxidation of membrane lipids can have numerous effects, including:

- increased membrane rigidity
- decreased activity of membrane-bound enzymes (e.g. sodium pumps)
- altered activity of membrane receptors.
- altered permiability

In addition to effects on phospholipids, radicals can also directly attack membrane proteins and induce lipid-lipid, lipid-protein and protein-protein crosslinking, all of which obviously have effects on membrane function.

Sources of free radicals

- Internal source
- External source
- Physiological factors

Internal sources

Some internal sources are mitochondria, phagocytes, xanthine oxidase, reactions involving iron and other transition metals, arachidonate pathways, peroxisomes, ischaemia, exercise and inflammation. These include enzymatic reactions involved in respiratory chain, in prostaglandin synthesis, in cytochrome p 450 system and in phagocytosis.

External sources

They are environmental pollutant, cigarette smoke, radiations, certain drugs, anesthetics, pesticides, industrial solvents and ozone. These can be non enzymatic reactions free radicals can also emerged from ionizing radiations.

Physiological factors

Disease status and mental conditions like stress and emotions can also form free radical.

Types of free radicals

- Superoxide radical
- Hydroperoxyl radical
- Hydrogen peroxide
- Triplet oxygen
- Active oxygen

Superoxide radical

It can oxidize sulphur, ascorbic acid and it can able to reduce metal ions and Cytochrome C. It can act as both oxidant and reactant. A reaction leads to the formation of hydrogen peroxide and oxygen is catalysed by superoxide dismutase.

Hydroperoxy radical

Formed by transfer of a proton to a oxygen atom. It is also called as perhydroxyl radical which is a protonated form of superoxide .

Hydrogen peroxide

It will act as a substrate in oxidation reaction involving synthesis of organic molecule. It is produced by univalent reduction of superoxide produces hydrogen peroxide and the effects are breaking up of DNA resulting in single strand breaks the formation of DNA protein cross link.

Triplet oxygen

Ions and elements are reacted with triplet oxygen to form oxides. It will form active peroxide radicals and it will undergo auto oxidation of unsaturated fattyacids.

Singlet oxygen

These are formed from hydrogen peroxide molecule. On decomposition it produces superoxide and hydroxyl radicals. It is not a free radical but it arises from some radical reactions.

Damages caused by free radicals

Inactivation of free radicals cause damage to all cellular macromolecules such as proteins, carbohydrates, lipids and nucleic acid and causes various diseases.

Oxidative damage to proteins and DNA

Oxidative destruction on protein results in site specific aminoacid modification, fragmentation of peptide chain, aggregation of cross linked reaction products, altered electrical charges and increased susceptibility to proteolysis. Oxidative attack on DNA results in base degradation, single strand breakage and cross link to proteins.

Free radical and diseases

Diseases like diabetes, hypertension, cancer, artherosclerosis, ischemia/reperfusion, inflammatory diseases(rheumatoid arthritis, pancreatitis and inflammatory bowel diseases), neurological diseases are caused by free radicals. Free radicals are not harmful always. To destroy invading pathogenic microbes which causes diseases, white blood cells release free radicals, thus sometime it is useful in the human body. Free radicals causes progressive adverse changes like aging pigments are stored in the subsarcolmal region of the muscle fibres which results in aging.

Mechanisms for Protection Against Radicals

Life on Earth evolved in the presence of oxygen, and necessarily adapted by evolution of a large battery of antioxidant systems. Some of these antioxidant molecules are present in all lifeforms examined, from bacteria to mammals, indicating their appearance early in the history of life.

Many antioxidants work by transiently becoming radicals themselves. These molecules are usually part of a larger network of cooperating antioxidants that end up regenerating the original antioxidant. For example, vitamin E becomes a radical, but is regenerated through the activity of the antioxidants vitamin C and glutathione.

Enzymatic Antioxidants

Three groups of enzymes play significant roles in protecting cells from oxidant stress:

Superoxide dismutases (SOD) are enzymes that catalyze the conversion of two superoxides into hydrogen peroxide and oxygen. The benefit here is that hydrogen peroxide is substantially less toxic that superoxide. SOD accelerates this detoxifying reaction roughly 10,000-fold over the non-catalyzed reaction.

SODs are metal-containing enzymes that depend on a bound manganese, copper or zinc for their antioxidant activity. In mammals, the manganese-containing enzyme is most abundant in mitochondria, while the zinc or copper forms predominant in cytoplasm. Interestingly, SODs are inducible enzymes - exposure of bacteria or vertebrate cells to higher concentrations of oxygen results in rapid increases in the concentration of SOD.

Catalase is found in peroxisomes in eucaryotic cells. It degrades hydrogen peroxide to water and oxygen, and hence finishes the detoxification reaction started by SOD.

Glutathione peroxidase is a group of enzymes, the most abundant of which contain selenium. These enyzmes, like catalase, degrade hydrogen peroxide. They also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants.

In addition to these enzymes, glutathione transferase, ceruloplasmin, hemoxygenase and possibly several other enzymes may participate in enzymatic control of oxygen radicals and their products.

Non-enzymatic Antioxidants

Three non-enzymatic antioxidants of particular importance are:

Vitamin E is the major lipid-soluble antioxidant, and plays a vital role in protecting membranes from oxidative damage. Its primary activity is to trap peroxy radicals in cellular membranes.

Vitamin C or ascorbic acid is a water-soluble antioxidant that can reduce radicals from a variety of sources. It also appears to participate in recycling vitamin E radicals. Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances.

Glutathione may well be the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (glutamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH.

In addition to these "big three", there are numerous small molecules that function as antioxidants. Examples include bilrubin, uric acid, flavonoids and carotenoids.

Reactive oxygen species

It includes oxygen radicals and several non radical oxidizing agents like hypochlorous acid, hydrogen peroxide, ozone etc. Reactive oxygen species have the tendency to donate oxygen to other species and it is responsible for the harmful effects of oxygen. They are highly reactive and unstable. Oxidative damage results in many diseases due to the presence of wide variety of oxygen free radicals and reactive species in the human body and food.

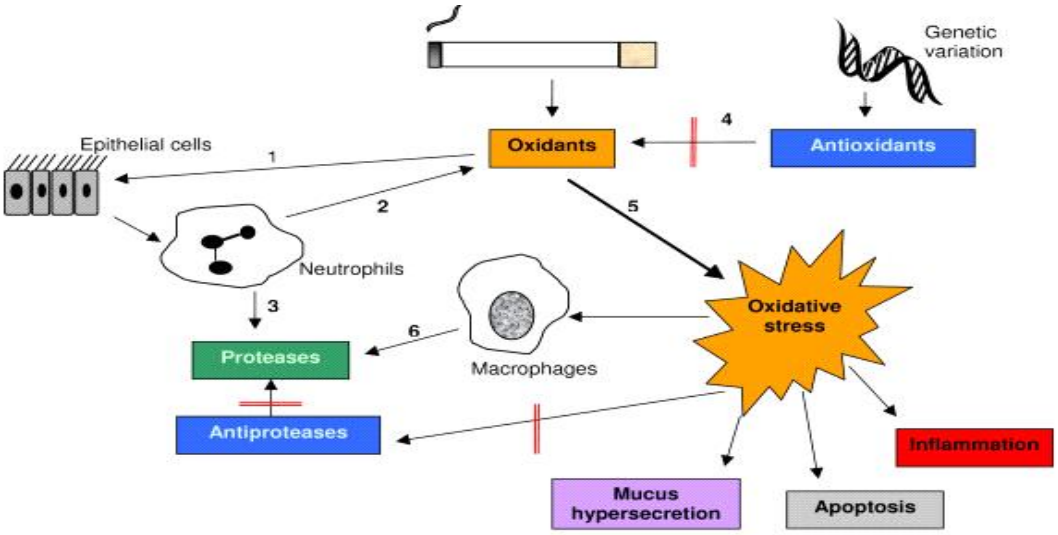
Reactive oxygen species include

- Hydroxyl radicals (-OH)
- Superoxide anions (O_2^-).
- Hydrogen peroxides **(H2O2)**
- Organic peroxides (R-OOH)
- Nitric oxide
- Singlet oxygen
- Peroxynitrite

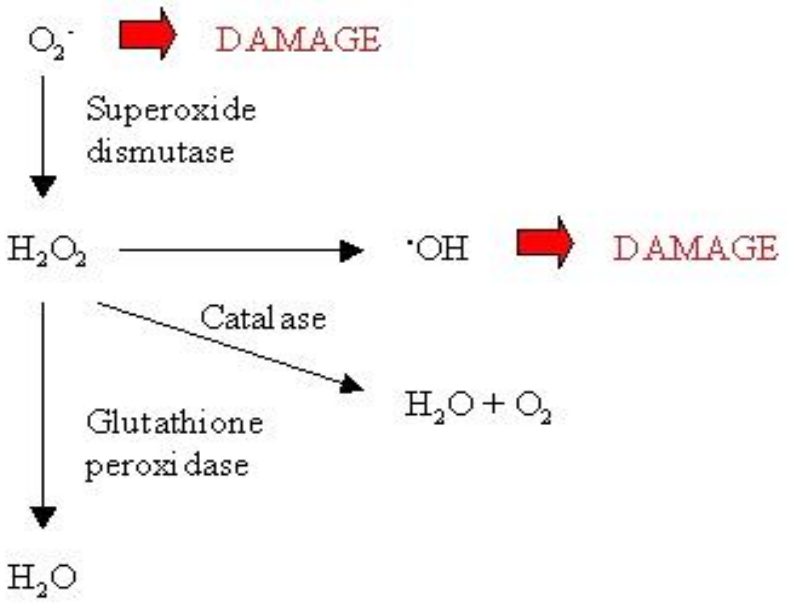
Oxidative stress and its effects

Simply oxidative stress is a damage made to a cell through oxidative process. Cells produce energy as a result of breathing, because of this activity highly reactive molecules called free radicals are formed. Oxidation is a normal process, but disturbances in that process such as attraction of free radical to a another molecule in the body results in toxic effects. The reactive oxygen species such as peroxides and free radicals are created from the metabolism of oxygen and they are generated by endogenous and exogenous process.

Figure: 1 Oxidants contained within cigarette smoke

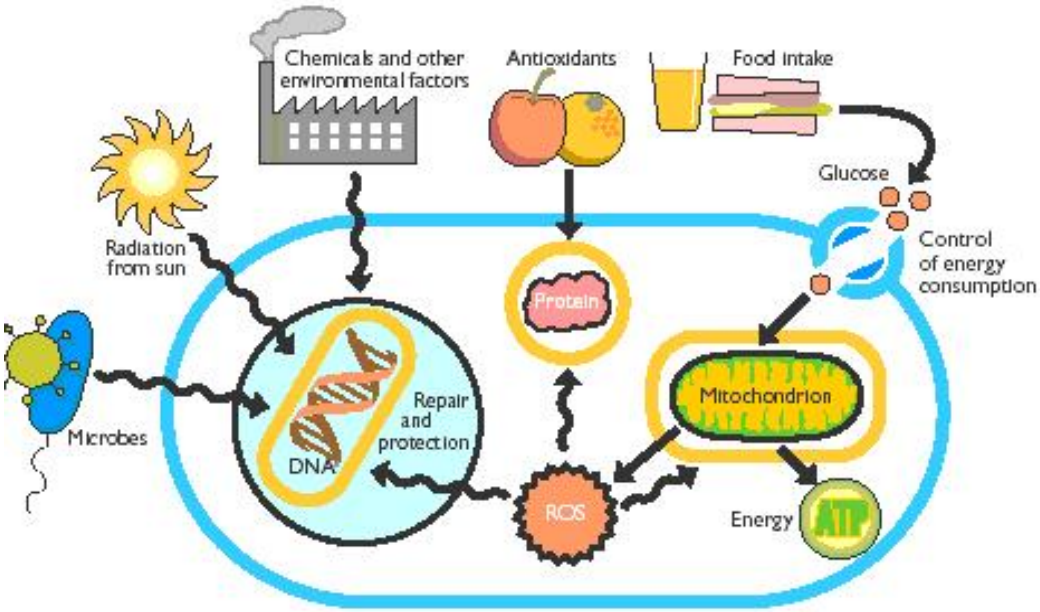


During oxidative cellular mechanism, hydrogen peroxide is produced that comes from breakdown of reactive oxygen species, the superoxide anion radicals (O_2^-). Superoxide is broken down into hydrogen peroxide and oxygen.Superoxide cause damage to the cells that produces mutations in the superoxide dismutase enzyme which leads to alanine transaminases (ALS), chracterised by loss of motornuerons in brainstem and spinalcord causes apoptosis through oxidative stress.



The complex network of antioxidant enzymes and metabolites joined together to prevent oxidative damage to cellular components such as DNA, lipids and proteins.

Figure: 2 Oxidative stress results



Oxidative stress and disease

Science has discovered that oxidative stress may cause more than seventy diseases. Oxidative stress is a common mechanism for the initiation and development of hepatic damage which leads to various liver disorders. Oxidative stress has major role in cardiovascular diseases. Low density lipoprotein oxidation trigger artherogenesis process which results in artherosclerosis and finally cardiovascular diseases. However antioxidant enzymes protects DNA from oxidative damage which cause cancer. So demand is great for the development of antioxidant agents. Diseases may vary depending on the toxins and stress in the body.

Some of the diseases caused by oxidative stress are:

- Cancer
- Lung disease
- Heart disease
- Arthritis
- Diabetis
- Fibromyalgia
- Autoimmune diseases
- Neurodegenerative diseases like parkinsonism and alzhemier's
- Eye diseases like macular degeneration

Antioxidant therapy has gained more important in the treatment of these diseases. Oxidative stress has an impact on body's aging process also. The decrease in melatonin levels seen with age correlates with an increase in neurogenerative

disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease and stroke, all disorders involve oxidative stress. In general, the production of Reactive oxygen species (ROS) increases with aging and is related with DNA damage to the tissues (**www.preventive /health/guide.com**).

Antioxidants

An antioxidant is a molecule which is capable of inhibiting the oxidation of other molecule. While oxidation reaction it transfer electrons or hydrogen atom from a substance to an oxidizing agent. Chain reactions are formed by the free radicals produced during oxidation reaction and it causes damage to the cell. By removing free radical intermediates, antioxidants inhibit oxidation reaction. Generally antioxidant system remove or prevent the reactive species, before they damage the cell components. The function of antioxidant is not to remove the entire oxidants but to keep at optimum level (**Docampo et al 1995**).

The interaction between different antioxidants with various metabolites and enzyme is having synergistic and interdependent effect on one another. The action of antioxidant is based upon the function of other members of antioxidant system. The protection provided by one antioxidant depend opon its concentration, its reaction towards particular reactive oxygen species and status of antioxidant with which it reacts. Some compounds produce antioxidant by chelating transition metal and preventing the formation of free radical.

Classification of antioxidants

- Natural antioxidants
- Synthetic antioxidants

Natural antioxidants

They are differ in their physical and chemical properties and composition, mechanism of action and their site of action. They are classified into following categories

Antioxidant enzymes

The antioxidant enzyme such as superoxide dismutase(SOD), catalase(CAT), glutathione peroxidase(GPx), glutathione reductase and glutathione transferase has an important role in destroying free radicals.

Superoxide dismutase (SOD) first reduces (adds an electron to) the radical superoxide ($O2^-$) to form hydrogen peroxide (H2O2) and oxygen (O2).

$2O2^- + 2H$ --SOD--> H2O2 + O2

Catalase and GPx then work simultaneously with the protein glutathione to reduce hydrogen peroxide and ultimately produce water (H2O).

2H2O2 --CAT--> H2O + O2

H2O2 + 2glutathione --GPx--> oxidized glutathione + 2H2O

(The oxidized glutathione is then reduced by another antioxidant enzyme -- glutathione reductase.)

Other enzyme act as secondary antioxidants to protect the cell from further damage.

Low molecular weight antioxidants

Through free radical scavenging property, it will delay or inhibit cellular damage. Two types of low molecular weight antioxidants are

- Lipid soluble antioxidants
- Water soluble antioxidants

Lipid soluble antioxidants

Carotenoids, tocopherol, quinones, bilirubin and polyphenols will come to this category. It will act against lipid peroxy radical as highly effective scavengers.

Lipid peroxy radical are formed as a result of free radical chain reaction of lipid peroxidation within lipoprotein.

Water soluble antioxidants

They are ascorbic acid, uric acid and polyphenols. It cannot act on the lipid moiety oflow density lipoprotein. It will support lipophillic antioxidants and regenerate them.

Synthetic antioxidants

They are approved by Food and Drug Administration. They are synthetic chemicals .Eg:

Butylated hydroxyl anisole (BHA), Butylated hydroxyl toluene (BHT), Tertiary butylated hydroxyl quinine (TVHQ).

Mechanism of action of antioxidants

It act by

- Scavenging initiating radicals eg:Action of superoxide dismutase in lipid phase to trap superoxide free radicals
- Reduction of concentration of reactive oxygen species, eg: Glutathione
- Chain breaking reaction. eg:Action of α-tocopherol in lipid phase to trap free radical
- By chelating transition meal catalyst eg: Action of group of compound by sequestering transition metals.

Regulation of antioxidant enzymes

The regulation of antioxidant enzymes mainly depends on the oxidant status of the cell, as it form the first line of defence against free radical. Enzyme modulating action of various hormones like growth hormone,prolactin and

melatonin are also involved in their regulation.Melatonsin is a derivative of aminoacid tryptophan, protect membrane lipids and nuclear DNA from oxidative damage. It has the ability to stimulate various antioxidant enzymes.

It will directly neutralize several reactive oxygen species including hydrogen peroxide, either by stimulating gene expression for the enzymes or by potentiating their activity.The reduction in enzyme activity was may also be due to reduction in their biosynthesis or due to their excessive utilization in trapping generated free radicles.It was also noticed that severe damage in liver decrease antioxidant defense in liver.Liver injury produce intracellular stress results in lipid peroxidation of membrane alomg with alteration of structural and functional characteristics of membrane results in altered function of antioxidant enzymes (**Halliwell et al 1999**).

Diethylnitrosamine

It is an N-nitroso alkyl compound which is a potent hepatotoxin and hepatocarcinogen, after repeated administration in experimental animals it causes tumors. Nitrosamines are compounds formed by the combination of amines and nitrates or nitrites (**Sivanesam karthikeyan et al 2010**). Many studies have recently shown that in gastric juice of human stomach, nitrosamines can be formed by a process called endogenous nitrosation. In many vegetables nitrates will be found, the bacteria in the mouth chemically reduce nitate to nitrite which can form nitrosating agents. In acidic environment of the stomach amines containing food that react with these nitrosating agents to form nitrosamines.

It can be seen in variety of products to which humans may exposed such as soyabean, cheese, salted and dried fish, cured meat, tobacco smoke, alcoholic bevareges and ground water. It can also seen in environment and can synthesis endogenously. Its exposure is dangerous to human population. Its metabolic activation is responsible for toxic effects, which results in release of highly reactive intermediates results in hepatocellular damage (**Kannampalli pradeep et al 2007**). Oxidative stress has a major role in diethylnitrosaamine induced hepatotoxicity. Many studies reported that continuous intrahepatic necroinflammatory changes were seen during liver damage induced by diethylnitrosamine.

Diethylnitrosamine undergo metabolic activation by cytochrome P 450 enzymes to form reactive electrophiles results in oxidative stress which further leads to cytotoxicity, mutagenicity and carcinogenicity. In the liver through an alkylated mechanism, diethylnitrosamine is hydroxylated by cytochrome P450 isoenzymes to become bioactive. Ethylation of the bases occurred as a result of reaction of bioactivated diethylnitrosamine with DNA. The ethyl DNA adducts interrupt base pairing results in mutation and activation of proto-oncogenes. Due to the generation of reactive oxygen species; it will initiate peroxidative damage to the cell and diethylnitrosamine will change antioxidant defense system in tissues.When the concentration of reactive oxygen species generated exceeds cell'ss antioxidant capability, oxidative damage to tissues or cells occurs. Diethylnitrosamine induced liver damage by enhancing monocytes / monocytes activation and eventual hepatocyte DNA damage. From the bioactivation of Diethylnitrosamine intermediate reactive compounds are originated , with important cell constituents it form covalent bonds, thus inducing mutation, cancer and necrosis.

The hepatocellular damage was observed histologically (thirty days after Diethylnitrosamine administration) with elevated levels of serum alkaline phosphatase, bilirubin,total protein, albumin, globulin and a simul;taneous fall in levels of marker enzymes in liver tissue. Oxidative stress of liver was confirmed by elevated levels of lipid peroxidation (LPO) as the membrane lipids are more susceptible to reactive oxygen species and decrease in enzymic and non-enzymic antioxidant activities.

Novel compounds are developing with antioxidant and hepatoprotective activity to treat or prevent cellular damage. Plant based medicines with good hepatoprotective activity are available and that can be used without any side effects. Many studies have showed that natural antioxidants will support the endogenous antioxidants defenses from reactive oxygen species ravage and by neutralizing free radicals it will restore optimum balance.

Chapter II

Literature Review

PLANT PROFILE

Botanical name(s)	:	Couroupita guianensis
Kingdom	:	Plantae
Division	:	magnoliophyta
Class	:	Dillenidae
Order	:	Lecythidales
Family	:	Lecythidaceae
Genus	:	Couroupita Aubl
Species	:	Couroupita guianensis Aubl
Popular name(s)	:	Nagalingam flowers, Shivalingam flowers
Parts used	:	Leaves, Flower, and Fruit

VERNACULAR NAMES

Hindi	:	Nagalinga flower
Tamil	:	Nagalingam flower
Telugu	:	Shivalinga flower or Nagamalli flower or Mallikarjuna flower
Kannada	:	Lingada mara
Marathi	:	Shivalingam flower
Bengali	:	Kaman gola

Common name

Cannon ball tree

ORGIN, DISTRIBUTION AND MORPHOLOGY

Cannon ball tree is native to rain forest of the guiana's in north eastern south America. Its a large deciduous topical tree. it possesses a dense, often narrow crown with leaves, clustered at the tip of branches. Leaves, upto 6"long are oval, oblong or broadly lance shaped with serrate margin. It flowers in racemes. The amazingly complex, yellow, reddish and pink flower of cannon ball tree are heavenly scented-a cross between a fine expensive perfume and a wonderful flower scent. These are 3" to 5" waxy pink and dark red flowers growing directly on the bark of the trunk. Flowers have six petals about 5cm and 2 inches long. They are large orange red, strongly perfumed. They are sterile, zygomorphic, and they have thick tangled extrusions that grow on a turnk. Flowering month is march to September.

The tree bears directly on the trunk and main branches, large globose woody fruits. They will be hanging in clusters, like balls on a string. The fruit contains small seeds in a white, unpleasant smelling white jelly, which are exposed when the upper half of the fruit goes off like a cover. The long dangling fruity branches give

the tree an unkempt appearance. It is pollinated by bats and they are very important for the survival of numerous species of plants. Although a plant of moist soils, it grows well under dry conditions..

CHEMICAL CONSTITUENTS

Flowers yield an aliphatic hydrocarbon, stigmasterol, alkaloids, phenolic, flavanoids, active principles like isatin and idirubin.It contains flavanoids-2'4'-dihydoxy-6-methoxy-3'5'-dimethyl chalcone,7-hydroxy-5-methoxy-6, 8-dimethyl flavone and the phenolic acid 4-hydroxy benzoic acid.

MEDICINAL USES

It is used as

- Antibiotic
- Antifungal
- Antiseptic
- Analgesic
- To cure colds

Juice from leaves is used for

- Inflammation
- Fever
- Alopecia
- Skin diseases
- Malaria

Fruit is used to

- Disinfect wounds
- Treat stomachache
- Cold
- malaria
- Toothache

Flower is used as

- Perfume
- Anti microbial

LITERATURE REVIEW

- **Mariana M.G. Pinheiro et al. ,(2010)** studied antinociceptive activity of crude ethanol extract and its fractions in three analgesic models(acetic acid-induced contortions, tail flick, and hot plate) from couroupita guianensis leaves. To elucidate mechanism of action from fractions, animals were pretreated (30 min) with atropine (muscarinic receptor antagonist,1mg/kg sc), mecamylamine (nicotinic receptor antagonist 2mg/kg sc), naloxone (opioid receptor antagonist 2mg/kg sc). Results showed all fractions produce antinociceptive activity in the tail flick model.Crude ethanol extracts and its fractions significantly inhibited number of contortions induced by acetic acid. Most prominent effect was observed in crude ethanol extract.

- **Sanjay Prahalad Umachigi et al., (2009)** evaluated antimicrobial, wound healing and antioxidant potential of *Couroupita guianensis* in rats. Ethanolic extract of whole plant *Couroupita guianensis* for the treatment of dermal wounds in rats was studied on excision and incision wound models. HPTLC of total extract was recorded for the purpose of standardization. Various parameters of wound incision, epithelization period , scar area , tensile strength and

hydroxyproline measurements along with wound contraction were used to evaluate the effect of *Couroupita guianensis* on wound healing. The results obtained showed that *Couroupita guianensis* accelerate the wound healing process by decrease in surface area of the wound and increase in tensile strength. Antimicrobial activity was studied against gram positive and gram negative bacteria compared to Erythromycin and Tetracycline. Moderate activity was observed against all organisms.

- **Ana Martinez et al., (2011)** has done protective effect against oxygen reactive species and skin fibroblast stimulation of *Couroupita guianensis* leaf extracts. Hydroalcoholic leaf extracts of *Couroupita guianensis* was examined for antioxidant activity, phytochemical and total phenolic composition , stimulation of skin fibroblast proliferation and UV absorption. The radical scavenging capacity , reducing power and protection against joint oxidation of linoleic acid and β-carotene bleaching oxidation in emulsion were used to evaluate the antioxidant activity. Result of the study strongly indicated that invitro antioxidant activity, which may be due to the presence of high total phenolic content. It also suggest that hydroalcoholic leaf extract of *Couroupita guianensis* have promising skin care properties.

- **V. Rajamanickam et al., (2009)** studied flower extracts of *couroupita guianensis* for invitro anthelmintic activity. Chloroform,acetone and ethanol extacts of flower of *couroupita guianensis* showed anthelmintic activity at a concentration of 50mg/ml and100mg/ml against adult earth worm pheritima posthuma.Activity was found to be increased according to the dose and compared with standard drug piperazine citrate.

- **M.R.Khan et al., (2008)** evaluated antibiotic acitivity of *Couroupita guianensis*. The result showed that methanolic extract of leaves, flowers, fruits ,stem and root bark has antibacterial and antifungal activity than aqueous and chloroform extract. It was found that klebsiella pneumonia and staphylococcus areus were the most susceptible bacteria and candida albicans and aspergillus fumigates were the susceptible fungi. Flowers , leaves, fruit pulp showed maximum antibacterial effect but bark of the plant showed least activity.

- **D.Pradhan et al., (2008)** has performed the immunomodulatory acitivity of the methanolic extract of *Couroupita guianensis* flower in rats. Successive methanolic extract of flowers of *Couroupita guianensis* showed significant immunostimulant activity on both the specific and non-specific immune mechanism. The results are encouraging enough to pursue bioactivity guided fractionation of this extract and structural elucidation of the active phytoconstituents.

- **Dr.Shaijesh Wankhede et al.,(2009)** evaluated the anxiolytic effect of methanolic root extract of *couroupita guianansis* in mouse.The effects of extracts on spontaneous activity and neuromuscular co-ordination were assessed.The result revealed that methanolic root extract showed good anxiolytic activity.

- **Sivanesan Karthikeyan et al.,(2006)** has showed that the Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Diethylnitrosamine induced hepatocellular damage was indicated by the elevated levels of serum aspartate transaminase , serum alanine transaminase and lipid peroxidation, and also the decrease in the levels of superoxide dismutase, catalase , glutathione peroxidase glutathione redutase and glutathione-s-transferase in the liver tissues. The results showed that the posttreatment with silymarin orally for 30 days exhibits a good hepatoprotective and antioxidant potential against diethylnitrosamine induced hepatocellular damage in rats.

- **Kannampalli Pradeep et al.,(2010)** evaluated the protective effect of Cassia fistula on diethylnitrosamine hepatocellular damage and oxidative stress in ethanol pretreated rats. The result suggest that oral administration of ethanolic leaf extract of Cassia fistula for 30 days to ethanol + diethlynitrosamine treated rats showed good hepatoprotective and antioxidant potential when compared to standard hepatoprotective drug ,silymarin.

- **Anupam Bishayee et al.,(2009)** evaluated resveratrol suppresses oxidative stress and inflammatory response in diethylnitrosamine-intiated rat hepatocarcinogenesis. They provide the evidence that attenuation of oxidative stress and suppression of inflammatory response mediated by Nrf2 (hepatic nuclear factor E2-related factor) showed chemopreventive effects against chemically-induced hepatic tumorigenesis in rats.

- **Malgorzata Kujawska et al., (2010)** investigated cloudy apple juice protect against chemically induced oxidative stress in rats. The cloudy apple juice exhibited very distinct protective effect on hepatic antioxidant enzymes. Results showed that protective action of apples phytochemicals by preventing damages of essential cellular macromolecules in the conditions of chemically induced oxidative stress in rats.

- **R . Gayathri et al., (2009)** evaluated ursolic acid attenuates oxidative stress mediated hepatocellular carcinoma induction by diethylnitrosamine in male Wistar rats. Antioxidant status was assessed by alterations in level of lipid peroxides and protein carbonyls. Oral administration ursolic acid 20mg/kg b.w for 6 weeks decreased the levels of lipid peroxides and protein carbonyls. The result showed the effectiveness of ursolic acid in reducing oxidative stress mediated changes in rats liver.

- **Sabry M Shaarawy et al.,(2009)** investigated protective effects of garlic and silymarin on diethylnitrosamine induced rats hepatotoxicity. Diethylnitrosamine increased oxidative stress, although administration of garlic or silymarin significantly reduced liver toxicity,combined administration was more effective in preventing hepatotoxicity. Hence they proved that garlic and silymarin have synergistic effect.

- **Ramanathan Sambath Kumar et al.,(2007)** evaluated antioxidant defense system in wistar albino rats assessed by the methanol extract of Bauhinia recemosa against diethylnitrosamine induced hepatocarcinogenesis. Diethylnitrosamine treated rats, significantly elevated levels of serum enzymes, bilirubin, and decreased levels of uric acid and protein were observed. Result

suggest that methanol extract of Bauhinia racemosa produced a protective effect by decreasing the level of serum enzymes, bilirubin, and increased protein and uric acid levels, it exert chemopreventive effect by suppressing nodule development and increasing the level of antioxidant.

- **Perumal Subramanian et al.,(2003)** evaluated S-Allylcysteine inhibits circulatory lipid peroxidation and promotes antioxidants in dietylnitrosamine-induced carcinogenesis. Result showed that rats treated with S-Allylcysteine showed inhibition of tumor incidence and lipid peroxidation with simultaneous elevation in antioxidants. Antioxidants level was enhanced by reducing the formation of free radicals.

- **Prasanna Galhana et al., (2009)** evaluated anti hepatocarcinogenic ayurvedic herbal remedy reduces the extent of diethylnitrosamine induced oxidative stress in rats. Results showed that treatment with decoction prepared from a mixture of nigella sativa seeds, hemidesmus indicus roots and smilax glabra rhizome-6gm/kg/day, for a period of ten weeks provide protection against diethylnitrosamine mediated changes in oxidative stress and produce anti hepatocarcinogenic effect.

- **Thamilarasan Manivasagam et al.,(2005)** studied the chemopreventive effect of diallyl disulphide on N-nitrosodiethylamine induced hepatocarcinogenesis. In N-nitrosodiethylamine treated rats,the levels of thiobarbituric acid substances and activities of superoxide dismutase and catalase were decreased whereas reduced glutathione and glutathione peroxidase were increased.Oral administration of diallyl disulphide(60mg/kg bodywt) produce chemopreventive effect by modulating the oxidant-antioxidant status of living system.

- **Nermin A.H. Sadik et al.,(2008)** studied the efficacy of dietary supplementation with blue berries on diethylnitrosamine-initiated hepatocarcinogenesis in male wistar rats.Results suggested that blue berries caused decreased in elevated serum levels of α-fetoprotein, homocysteine, glutathione, deoxyribonucleic acid, ribonucleic acid, and activity of glutathione reductase in liver and

histopathological damage was minimized in that group. It was documented that blue berries was a chemopreventive natural supplement for liver cancer.

- **Mohamed. M. Sayed-ahmed et al.,(2010)** evaluated thymoquinone attenuates diethylnitrosamine induction of hepatic carcinogenesis through antioxidant signaling. Results showed that thymoquinone supplementation prevents the development of diethylnitrosamine induced liver cancer by decreasing oxidative stress and preserving both the activity and mRNA expression of antioxidant enzymes.

Chapter III

Aim and Objective

AIM AND OBJECTIVE OF STUDY

Diethylnitrosamine is an N-nitrosoalkyl compound, categorized as a potent hepatotoxin and hepatocarcinogen in experimental animals (**Jose et al., 1998**). The main cause for concern is that diethylnitrosamine is found in a wide variety of foods like cheese, soyabean, smoked, salted and dried fish, cured meat and alcoholic beverages (**Liao et al., 2001**). Metabolism of certain therapeutic drugs is also reported to produce diethylnitrosamine (**Akintonwa, 1985**). It is also found in tobacco smoke at a concentration ranging from 1 to 2ng/cigarette and in baby bottle nipples at a level of 10 ppb (**IARC, 1972**). Diethylnitrosamine is reported to undergo metabolic activation by cytochrome P450 enzymes to form reactive electrophiles which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (**Archer, 1989**). The detection of diethylnitrosamine in commonly consumed food products makes the human population vulnerable to its exposure.

As oxidative stress plays a central role in diethylnitrosamine induced hepatotoxicity, the use of antioxidants would offer better protection to counteract liver damage (**Vitaglione et al., 2004**). This constraint underscores the need for the development of novel potent antioxidant property. Since modern medicines have little to offer for alleviation of oxidative stress in hepatic diseases, plant based preparations are employed in treatment of liver disorders. Number of medicinal plants have shown antioxidant and hepatoprotective activity due to the presence of active constituents. From the literature survey, it was found that the flower of C ouroupita guianensis is rich in flavonoids, phenolic compounds and alkaloids. It was also noticed that Couroupita guianensis is used as antimicrobial, antiseptic, analgesic, anti-inflammatory, antipyretic, antimalarial etc. This widespread use of Couroupita guianensis in traditional medicine promoted me to evaluate the antioxidant status of flower extract in chemically induced hepatic injury in albino rats. Since oxidative stress plays a main role in hepatic injury which further leads to hepatocarcinogenesis, N-diethylnitrosamine which cause oxidative injury and hepatocarcinogenesis, is selected for the study.

Chapter IV

Plan of Work

PLAN OF WORK

Collection

Collection of Couroupita guianensis flower, authentification and shade drying.

Extraction

Extraction of powdered flower material with petroleum ether followed by ethanol.

Phytochemical examination for identification of chemical constituents

Pharmacological evaluation:

Acute oral toxicity study of ethanol extract of *Couroupita guianensis* flower extract (OECD Guideline 425)

Evaluation of *Couroupita guianensis* flower extract on N-diethylnitrosamine induced oxidative stress induced oxidative stress in liver.

Parameters Considered for evaluation

Liver function test

SGOT
SGPT
ALP
TOTAL PROTEIN
TOTAL BILIRUBIN
ALFA FETOPROTEIN
CARCINOEMBRYONIC ANTIGEN

Evalution of liver oxidant–antioxidant status in liver

Superoxide dismutase (SOD)

Catalase (CAT)

Glutathione Peroxidase (GPx)

Glutathione S-Transferase (GST)

Glutathione reductase (GR)

Lipid peroxidation (LPO)

Histopathological examination of liver

Chapter V

Materials and Method

MATERIALS AND METHODS

Chemicals:

Diethylnitrosamine is purchased from sigma Aldrich chemical company, (St. Louis, MO, USA) petroleum ether and ethanol was purchased from Nice chemicals Pvt Ltd. Silymarin was obtained as gratis from Himalaya drug company, bengaluru, india. All other chemicals used were of analytical grade and were purchased locally.

PLANT MATERIAL

The flowers of couroupita guianensis were collected from the botanical garden, J. K. K. Nattraja college of pharmacy, Komarapalayam. The flowers were taxonomically identified, confirmed and authenticated by the botanical survey of India, souther circle, Tamilnadu agricultural university, Coimbatore with authentication number BSI/SRC/5/23/2011-12/TECH-883. The voucher specimen was retained in our laboratory for further reference.

EXTRACTION

The collected flowers were shade dried completely. The dried material was then coarsely powdered and was sieved (sieve # 40) to get uniform coarse powder.

The dried coarse powder was defatted with petroleum ether (60 - 80°c) in a soxhlet extractor in order to remove fatty substances, which may interfere with the isolation of chemical constituents. The defatted marc was dried and it was subjected to extraction with ethanol (95%) in a soxhlet apparatus for 72 hours.The solvent was then distilled off and the extract obtained was concentrated to dryness under reduced pressure and percentage yield was calculated.

PHYTOCHEMICAL SCREENING

The extract obtained was subjected to Priliminary Phytochemical screening (Khandelwal and Kokate, 1995).

Test for alkaloids:

Small of extract was dissolved in 10 ml of 0.1N dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids.

Mayer's test

Filtrate was treated with Mayer's reagent. Formation of yellow cream precipitate indicates the presence of alkaloids.

Dragendroff's test

Filtrate was treated with Dragendroff's reagent. Formation of red coloured precipitate indicates the presence of alkaloids.

Hager's test

Filtrate was treated with Hager's reagent. Formation of yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test

Filtrate was treated with wagner's reagent. Formation of brown (or) reddish brown precipitate indicates the presence of alkaloids.(**Rosenthalar, 1930**)

Detection of Phytosterols and Triterpenoids :

0.5 gm of extract was treated with 10ml of chloroform and filtered. The filtrate was used to test the presence of phytosterols and Triterpenoids.

Libermann's Test

To 2 ml filtrate in hot alcohol, few drops of acetic anhydride were added. Formation of brown precipitate indicates the presence of sterols.

Libermann's Burchard Test

100 mg of extract was treated with 2 ml of chloroform and filtered. To the filtrate few drops of acetic anhydride was added, boiled and cooled. Concentrated H_2So_4 was added through the sides of the test tube. Formation of brown ring at the junction indicates the presence of steroidal saponins.

Salkowski Test

To the test extract solution few drops of Concentrated H_2So_4 was added, shaken and allowed to stand, lower layer turns red indicates the presence of sterols. (**Peach and Tracey, 1957**)

Detection of Flavoniods :

Shinoda Test

To 100 mg of extract, few fragments of magnesium metal were added in a test tube, followed by drop wise addition of concentrated hydrochloric acid. Formation of magenta colour indicates the presence of Flavonoids.

Alkaline Reagent Test

To 100 mg of extract, few drops of sodium hydroxide solution were added in a test tube. Formation of intense yellow colour that becomes colourless on addition of few drops of dilute hydrochloric acid indicates the presence of flavanoids. (**Shellard, 1957**)

Detection of Saponins :

Foam test

The extract was diluted with 20 ml of distilled water and it was shaken in a graduated cylinder for 15 minutes. A 1cm layer of foam indicates the presence of Saponins.

Detection of Proteins and Amino acids:

100 mg of extract was taken in 10 ml of water and filtered. The filtrate was used to test the presence of protein and amino acids.

Millon's Test

2 ml of filtrate was treated with 2 ml of millon's reagent in a Test tube and heated in a water bath for 5 minutes, cooled and few drops of $NaNo_2$ were added. Formation of white precipitate, which turns to red upon heating, indicates the presence of proteins and amino acids.

Ninhydrin Test

2 ml of filtrate, 0.25% ninhydrin reagent was added in a test tube and boiled for 2 minutes. Formation of blue colour indicates the presence of amino acids.

Biuret Test

2 ml of filtrate was treated with 2 ml of 10% sodium hydroxide in a test and heated for 10 minutes. A drop of 7% copper sulphate solution was added in the above mixture. Formation of purplish violent indicates the presence of proteins.

Detection of Fixed oils and Fats:

Oily Spot Test

One drop of extract was placed on filter paper and the solvent was evaporated. An oily stain of filter paper indicates the presence of fixed oil. (**Rosenthalar, 1930**)

Detection of Phenolics and Tannins:

100 mg of extract was boiled with 1ml of distilled water and filtered. The filtrate was used for the test.

Ferric chloride Test

To 2 ml of filtrate, 2ml of 1% ferric chloride was added in a test tube. Formation of bluish black colour indicates the presence of phenolic nucleus.

Lead acetate Test

To 2 ml of filtrate, few drops of lead acetate solution were added in a test tube. Formation of yellow precipitate indicates the presence of tannins.

Detection of Carbohydrate:

500 mg of extract was dissolved in 5ml of distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

Molisch test

To one ml of filtrate, two drops of Molisch reagent was added in a test tube and 2 ml of concentrated H_2So_4 was added carefully along the side of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Fehling's test

To one ml of filtrate, 4 ml of fehling's reagent was added in a test tube and heated for 10 minutes in a water bath. Formation of red precipitate indicates the presence of reducing sugar.

Benedict's test

Filtrate was treated with Benedict's reagent and heated on water bath. Formation of orange red precipitate indicates the presence of reducing sugars.

Detection of Glycosides:

0.5 gm of extract was hydrolyzed with 20 ml of 0.1N dilute hydrochloric acid and filtered. The filtrate was used to test the presence of glycosides.

Modified Borntrager's test

1 ml of filtrate 2 ml of 1% ferric chloride solution was added in a test tube and heated for 10 minutes in boiling water bath. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with half its volume of ammonia solution. Formation of rose pink or cherry colour in the ammonical layer indicates the presence of anthranol glycoside.

Legal's test

To 1 ml of filtrate, 3 ml of sodium nitroprusside in pyridine and methanolic alkali (KOH) was added in a test tube. Formation of pink to blood red colour indicates the presence of cardiac glycoside.

Keller Killiani Test

Small portion from the extract was shaken with 1ml of Glacial acetic acid containing trace of ferric chloride. 1 ml of concentrated H_2So_4 was added carefully by the sides of the test tube. A blue colour in the acetic acid layer and red colour at the junction of two liquids indicate the presence of glycosides. (**Rosenthalar, 1930**).

PHARMACOLOGICAL SCREENING

Acute oral toxicity study of Couroupita guianensis flower extract

Animals

Swiss albino mice of female sex weighing 20-25gms were used for the study. The animals were obtained from Agricultural University, Mannuthy, Thrissur, kerala (328/99/CPCSEA) and were housed in polypropylene cages. The animals were maintained under standard laboratory conditions (25^0 + 2^0C; 12hr light and dark cycle). The animals were fed with standard diet and water *ad libitum*. Ethical clearance (for handling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee (887/ac/05/CPCSEA) before performing the study on animals. The proposal number is 31MP15JUN11

Acute oral toxicity study

Acute oral toxicity study of Couroupita guianensis flower extract was carried out as per OECD guideline 425 (Up and Down procedure). The test procedure minimizes the number of animals required to estimate the acute oral toxicity. The test allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Animals were fasted (food but not water was with held overnight) prior to dosing. The fasted body weight of each animal was determined and the dose was calculated according to the body weight.

Limit test at 2000mg/kg

The extract was administered in the dose of 2000mg/kg body weight orally to one animal. If the first test animal survives, then four other animals were dosed sequentially; therefore, a total of five animals were tested. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hour), and daily thereafter, for a total of 14 days. After the experimental period, the animals were weighed and humanely killed and their vital organs including heart, lungs, liver, kidneys, spleen, adrenals, sex organs and brain were grossly examined **(OECD Guidance; 2000)**

Effect of Couroupita guianensis flower extract on N-diethylnitrosamine induced hepatic damage in wistar rats.

ANIMALS:

Experiments were carried out according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals, New Delhi, India. The protocol of experiments were approved by Institutional Animal Ethics Committee (IAEC) (887/ac/CPCSEA), J.K.K. Nattraja college of pharmacy, Komarapalayam, Nammakal district.

Male wistar rats weighing about 100 -200gms, were obtained from agricultural university, mannuthy, Thrissur. The animals were maintained in animal house under standard environmental condition ($25^0 \pm 2^0$c) and 12hr/12hr light and dark cycle. Animals were fed with standard pellet diet (Hindustan Lever Ltd, mumbai, india) and water *ad.libtum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and experiments were conducted according to the CPCSEA, India guidelines on the use and care of experimental animals.

PROCEDURE

Total 30 animals were used for this study and it was divided into 5 groups of 6 animals each.

Group I : Rats served as controls received normal saline 1ml/kg (i.p.) on day 0 and carboxymethylcellulose (2ml/kg, orally) for 30 days.

Group II : Rats were administered with a single dose of Diethylnitrosamine (200 mg/kg b.w , i.p.) in saline on day 0 and 0.5% w/v carboxymethylcellulose 2ml/kg (orally) from day 1 to 30.

Group III : Rats were administered with diethylnitrosamine (200 mg/kg b.w.,i.p) in saline on day 0 followed by extract (200 mg/kg.,p.o) in carboxymethylcellulose from day 1 to 30.

Group IV : Rats were administered with diethylnitrosamine (200 mg/kg b.w.,i.p) in saline on day 0 followed by extract (400 mg/kg.,i.p) in carboxymethylcellulose from day 1 to 30.

Group V : Rats were administered with diethylnitrosamine (200mg/kg.,i.p) in saline on day 0 followed by silymarin (50 mg/kg b.w.,p.o.) in carboxymethylcellulose from day 1 to 30.

At the end of experimental period, blood sample was collected from retro-orbital plexus under anaesthesia and serum was seperated by centrifugation, which was subjected to biochemical analysis.

Animals were sacrificed by cervical decapitation and the liver was excised, washed in ice cold saline and blotted to dryness. A 1% homogenate of the liver tissue was prepared in Tris-Hcl buffer (0.1M; PH 7.4), centrifuged at 1000 rpm for 10 minutes at 4°c to remove the cell debris. The clear supernatant is used for further biochemical assays (Pradeep et al., 2007).

ASSESSMENT OF HEPATOPROTECTIVE ACTIVITY

- Morphological parameters
- Biochemical parameters

BIOCHEMICAL PARAMETERS:

Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase(ALP), Albumin (ALB), Globulin (GLO), Total protein (T.PRO), Total bilirubin (T.B), Direct bilirubin (D.B), Indirect bilirubin .(I.B), Alpha fetoprotein (AFP), Carcino embryogenic antigen (CEA) were analysed in serum. Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), Glutathione-S- transferase (GST), Glutathione reductase (GR), Lipid peroxidation (LPO), Vitamin C, Vitamin E were analysed in liver tissue.

Histopathology: Immediately after blood collection the animals were sacrificed and the liver was collected and fixed in 10% neutral formalin. The tissues were then embedded in molten paraffin wax and were ultra sectioned (5-6µm thickness), stained with hematoxylin and eosin and were examined under light microscope for histopathological changes (**Amit Khatri et al., 2009**).

Satistical analysis

Results were expressed as mean ± standard error of mean(SEM). The results were analysed for statistical significance by one way ANOVA followed by dunnett's test (Graphpad Software Inc,La Jolla, CA. Trial version). The criterion for statistical significance was set at $p < 0.05$.

Chapter VI

Results and Discussion

TABLE.1 Percentage yield

Petroleum ether	2.97% w/w
Ethanol	7.14 % w/w

TABLE.2 Phytoconstituents detected in of *Couroupita guianensis* flower extract

TEST	Phytoconstituents Detected
Test for Alkaloids	
Mayer's test	+
Dragendroff's	+
Hager's test	+
Wagner's test	+
Test for Flavonoids	
Alkaline reagent test	+
Test for Saponins	
Foam test	-
Test for Proteins and Aminoacids	
Millon's test	+
Ninhydrin test	+
Biuret test	+
Test for Phenolics and Tannins	
Ferric chloride test	+
Lead acetate test	+
Test for carbohydrates	
Molisch's test	+
Fehling's test	+
Benedict's test	+
Test for Gylcosides	
Modified Borntrager's test	+
Legal's test	+
Keller-Killiani test	+

\+ = Present

\- = Absent

TABLE.3 Acute oral Toxicity study (425) observations.

RESPIRATORY BLOCKAGE IN NOSTRIL	
Dyspnoea	Nil
Apnoea	Nil
Tachypnea	Nil
Nostril discharge	Nil
MOTOR ACTIVITIES	
Locomotion	Normal
Somnolence	Nil
Loss of righting reflex	Nil
Anaesthesia	Nil
Catalepsy	Nil
Ataxia	Nil
Toe walking	Nil
Prostration	Nil
Fasciculation	Nil
Tremor	Nil
CONVULSION (INVOLUNTRAY CONTRACTION)	
Clonic/tonic/tonic-clonic convulsion	Nil
Asphyxial convulsion	Nil
Opistotones (titanic spasm)	Nil
REFLEXES	
Corneal	Normal
Eyelid closure	Normal

Righting	Normal
Light	Normal
Auditory and sensory	Normal
OCULAR SIGNS	
Lacrimation	Nil
Miosis	Nil
Mydriasis	Nil
Ptosis	Nil
Chromodacryorrhea	Nil
Iritis	Nil
Conjunctivitis	Nil
SALIVATION	
Saliva secretion	Nil
PILOERECTION	
Contraction of erectile tissue	Nil
ANALGESIA	
Decrease in reaction to induced pain	Nil
MUSCLE TONE	
Hypo or hypertonia	Nil
GIT SIGN	
Solid dried / watery stool	Nil
Emesis	Nil
Red urine	Nil
SKIN	
Oedema	Nil

TABLE.4 Effect of *Couroupita guianensis* flower extract on α-feto protein and Carcino embryonic antigen (CEA) level in serum of control and experimental group rats.

GROUP	DOSE	AFP (ng/ml)	CEA (ng/ml)
Control	-	10.25±0.256	11.67±1.014
DEN	200mg/kg	29.55±2.307***[a]	29.03±2.139***[a]
DEN + Extract	200mg/kg	23.03±1.426*[b]	20.80±0.481***[b]
DEN + Extract	400mg/kg	17.52±0.634***[b]	17.51±0.464***[b]
DEN + Silymarin	50mg/kg	14.37±0.744***[b]	15.47±1.152***[b]

All values are expressed as mean±S.E.M, n=6 in each group.

[a] values are significantly different from control group; ns-non significant; *p < 0.05; **p < 0.01; ***p < 0.001.

[b]values are significantly different from DEN- induced group; ns-non significant;*p<0.05;**p<0.01;***p<0.001.

Figure.1 Alpha- feto protein

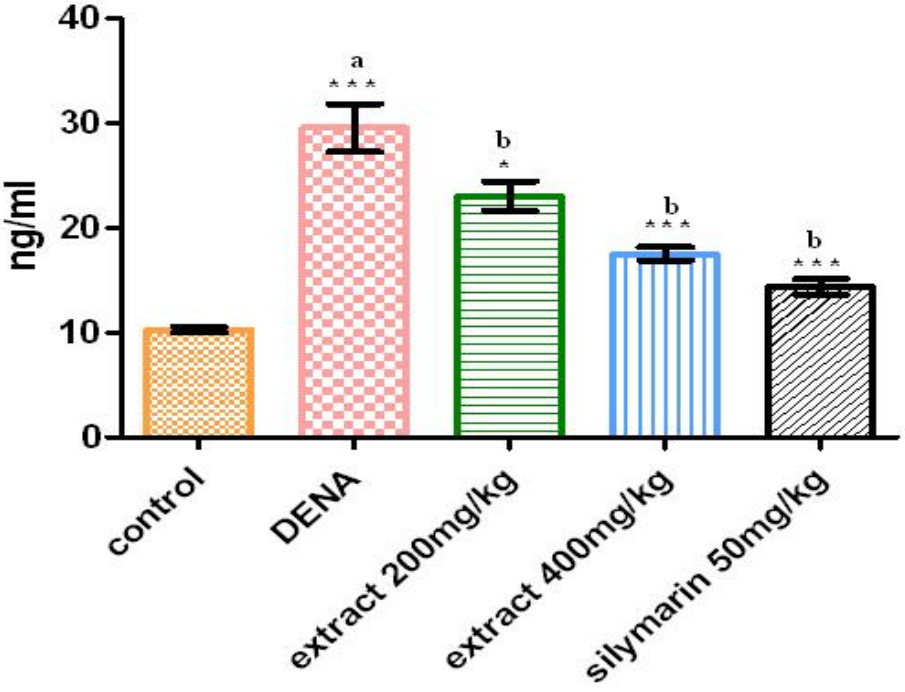


Figure.2 Carcinoembryonic antigen

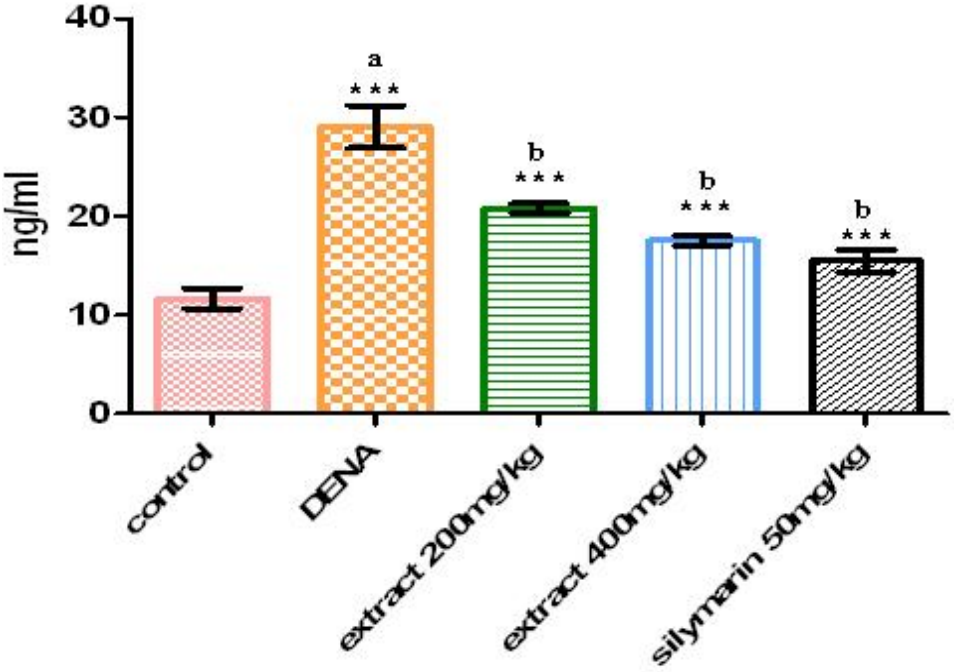


TABLE.5 Effect of *Couroupita guianensis* flower extract on the activities of marker enzymes in the serum of control and experimental groups of rats

GROUP	DOSE	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TOTAL BILIRUBIN (mg/dl)	TOTAL PROTEIN (mg/dl)
Control	--	31.83±1.249	30.33±1.520	5.00±2.145	0.433±0.021	10.75±0.214
DEN	200mg/kg	49.33±1.687***[a]	40.50±0.562***[a]	141.7±3.333***[a]	0.791±0.020***[a]	7.767±0.091***[a]
DEN + Extract	200mg/kg	41.50±1.746**[b]	34.50±1.688*[b]	124.7±4.088**[b]	0.633±0.021***[b]	8.417±0.090*[b]
DEN + Extract	400mg/kg	35.33±1.202***[b]	32.67±0.666**[b]	105.7±3.765***[b]	0.500±0.025***[b]	9.267±0.158***[b]
DEN + Silymarin	50mg/kg	38.00±0.730***[b]	31.67±2.044***[b]	96.67±1.542***[b]	0.516±0.0166**[b]	10.17±0.197***[b]

All values are expressed as mean±S.E.M, n=6 in each group.

[a] values are significantly different from control group; ns-non significant; *p < 0.05; **p < 0.01; ***p < 0.001.

[b]values are significantly different from DEN- induced group; ns-non significant;*p<0.05;**p<0.01;***p<0.001.

Figure. 3 SGOT

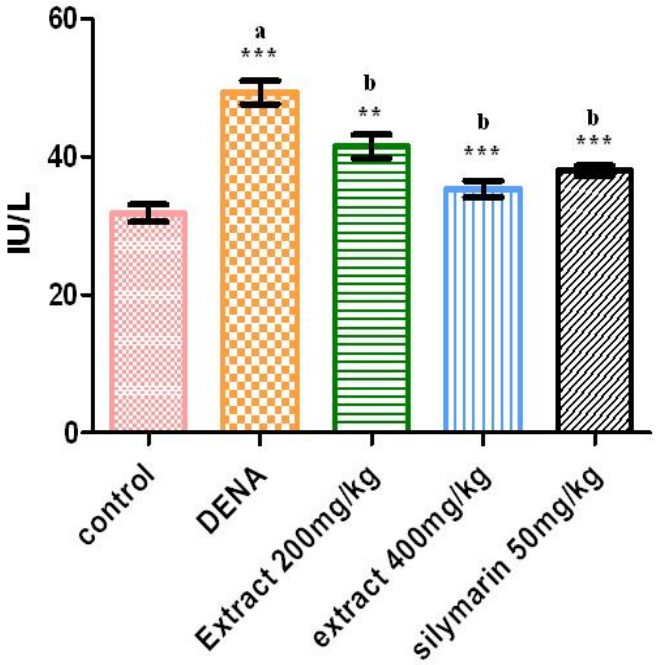


Figure. 4 SGPT

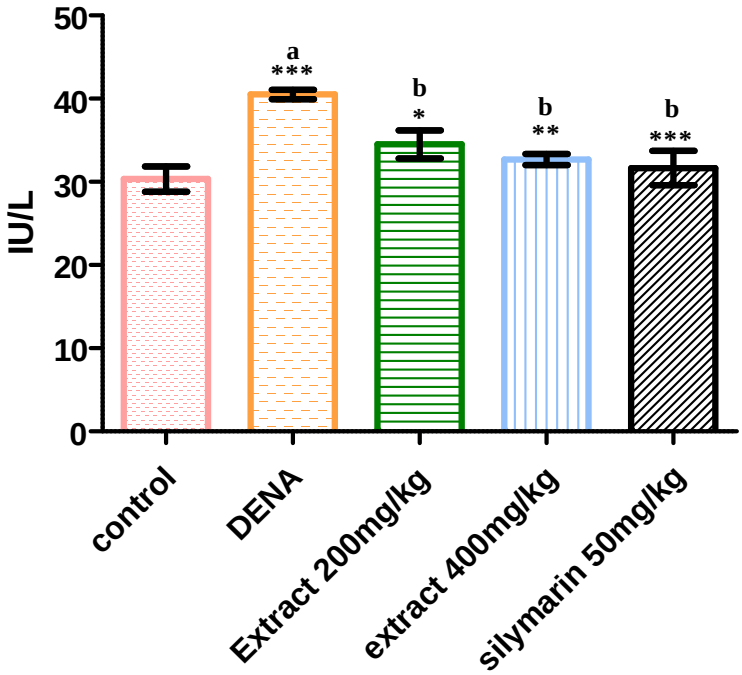


Figure. 5 ALP

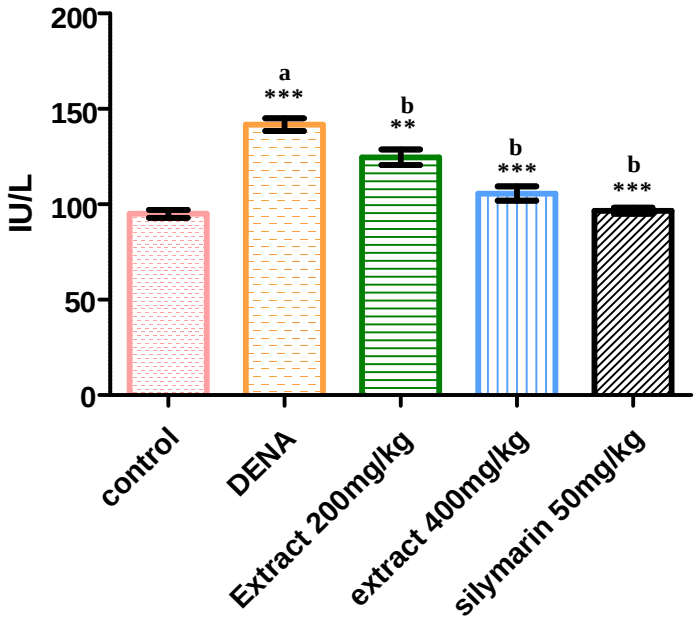


Figure. 6 BILIRUBIN

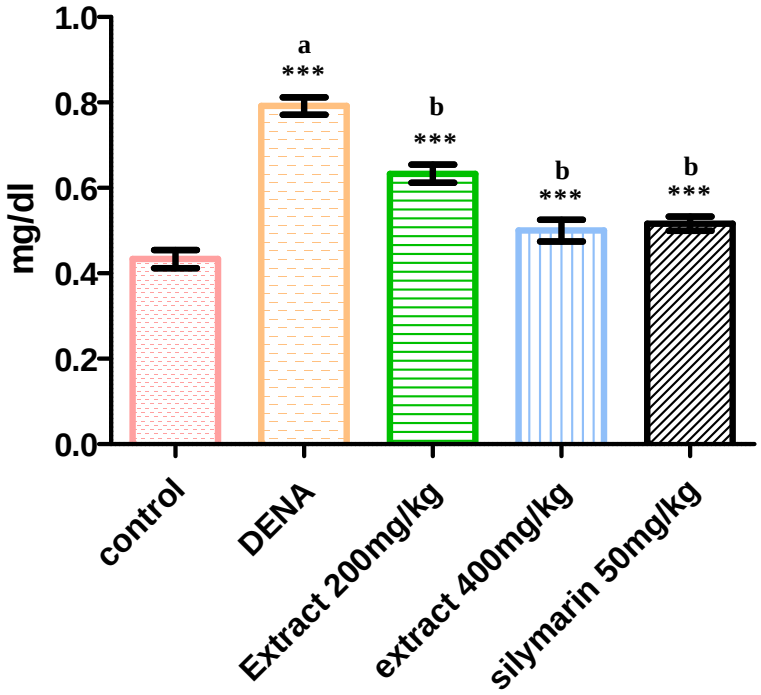


Figure. 7 PROTEIN

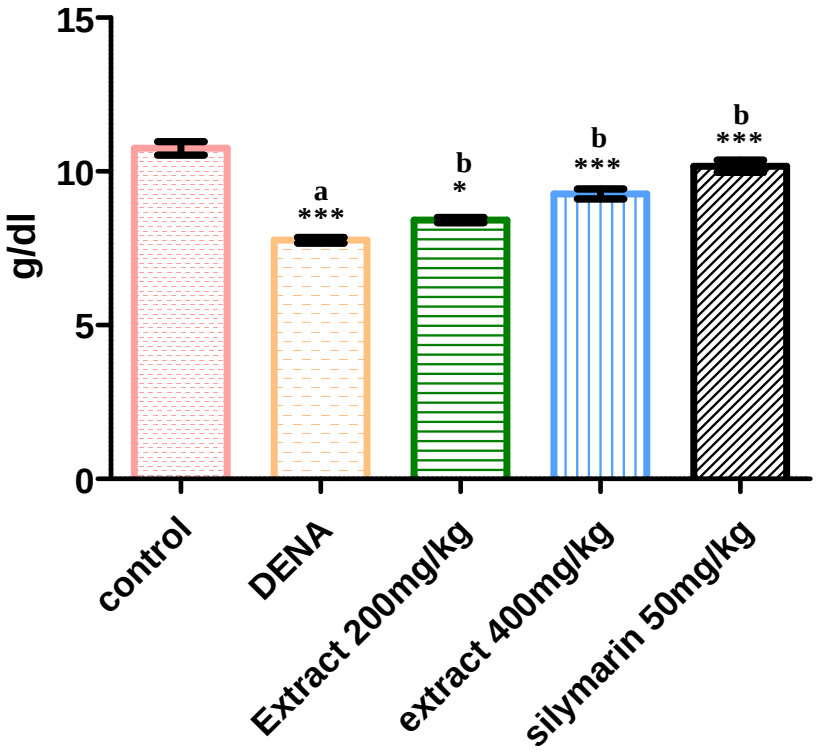


TABLE.6 Effect of *Couroupita guianensis* flower extract on liver oxidant–antioxidant status in liver tissue of control and experimental groups of rats

GROUP	DOSE mg/kg	SOD Unit/mg of protein	CATALASE Unit/mg of protein	GPX Unit/mg of protein	GST Unit/mg of protein	GR µmole of NADPH oxidized/(min mg protein)	LPO MDA umol/hr/gr of tissue
Control	--	1.550±0.0341	1.400±0.0516	1.650±0.0562	0.9467±0.024	1.300±0.051	0.583±0.02
DEN	200mg/kg	0.683±0.0401***[a]	0.801±0.0240***[a]	1.115±0.0320***[a]	0.6550±0.014***[a]	0.815±0.060***[a]	0.891±0.02***[a]
DEN + Extract	200mg/kg	1.050±0.0562**[b]	0.881±0.0370 ns [b]	1.098±0.0271 ns [b]	0.8133±0.013***[b]	1.052±0.020**[b]	0.628±0.03***[b]
DEN + Extract	400mg/kg	1.467±0.0557***[b]	1.013±0.0477**[b]	1.367±0.055**[b]	0.8767±0.009***[b]	1.217±0.047***[b]	0.601±0.010***[b]
DEN + Silymarin	50mg/kg	1.450±0.0922***[b]	1.300±0.0365***[b]	1.600±0.063***[b]	0.8950±0.033***[b]	1.283±0.040***[b]	0.596±0.02***[b]

All values are expressed as mean±S.E.M, n=6 in each group.

[a] values are significantly different from control group; ns-non significant; *p < 0.05; **p < 0.01; ***p < 0.001.

[b] values are significantly different from DEN- induced group; ns-non significant;*p<0.05;**p<0.01;***p<0.001.

Figure. 8 SOD

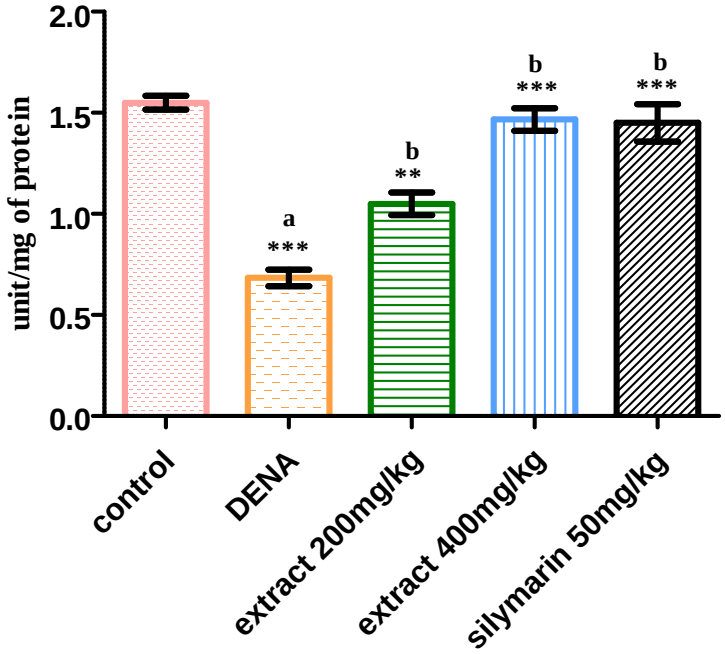


Figure. 9 CATALASE

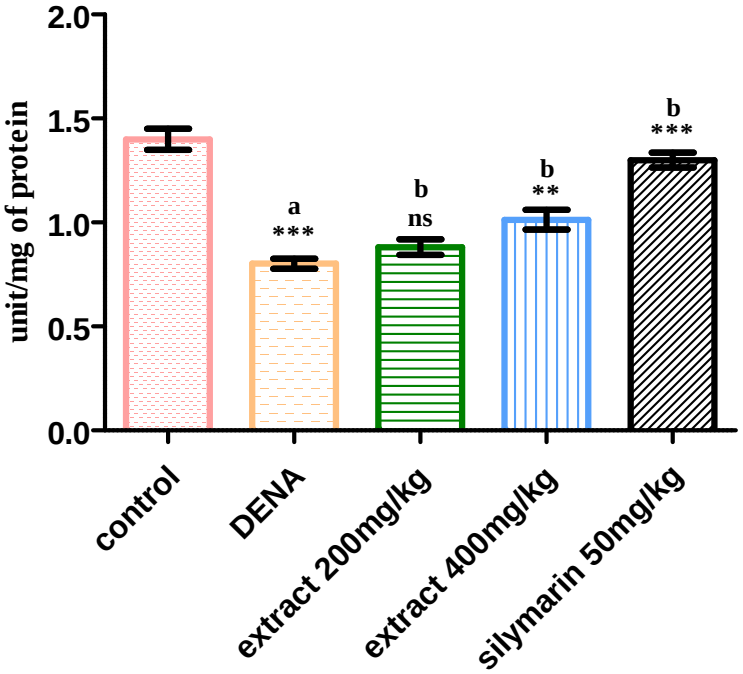


Figure. 10 GPX

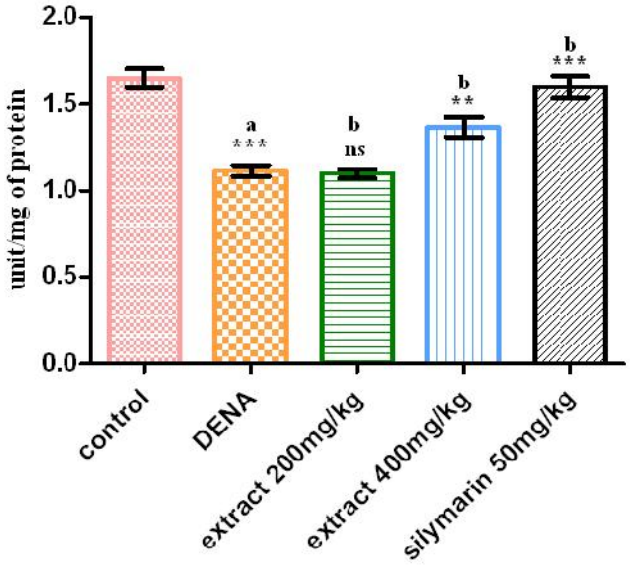


Figure. 11 GST

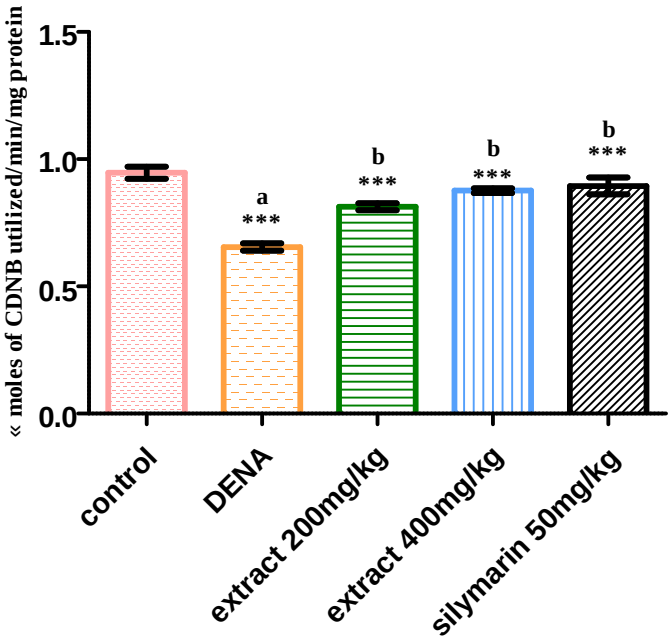


Figure. 12 GR

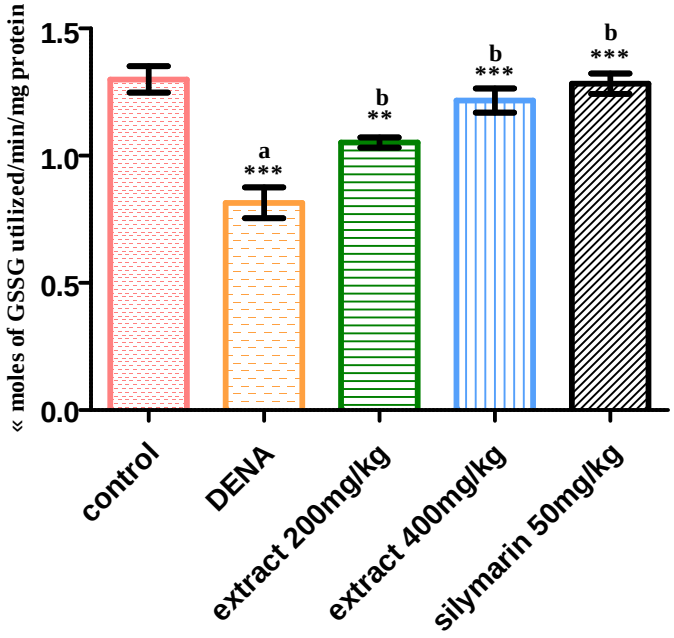


Figure. 13 LPO

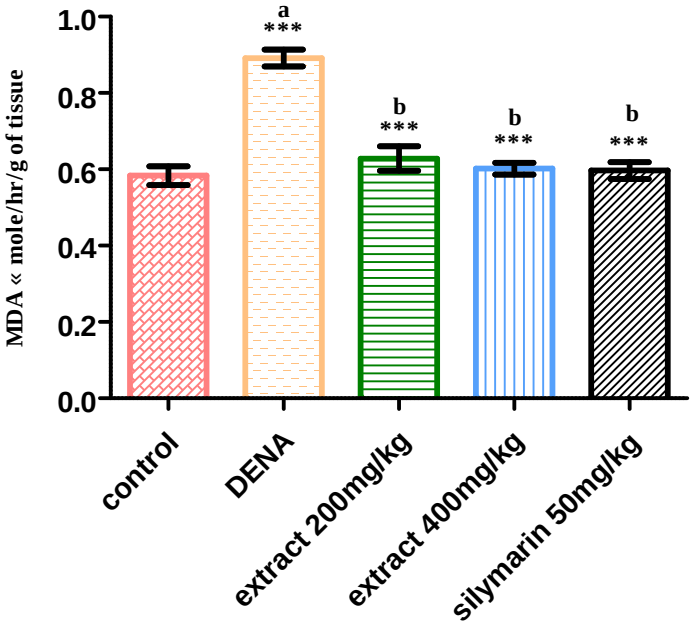


TABLE.7 Effect of *Couroupita guianensis* flower extract on the levels of vitamin-C and vitamin-E in liver tissue

GROUP	DOSE (mg/kg)	VITAMIN – C (mg/gm wet tissue)	VITAMIN – E (mg/gm wet tissue)
Control	--	9.483±0.204	1.150±0.0223
DEN	200mg/kg	7.083±0.047***[a]	0.683±0.040***[a]
DEN + Extract	200mg/kg	8.35±0.095***[b]	0.983±0.047***[b]
DEN + Extract	400mg/kg	8.650±0.095***[b]	1.100±0.044***[b]
DEN + Silymarin	50mg/kg	8.533±0.210***[b]	1.183±0.040***[b]

All values are expressed as mean±S.E.M, n=6 in each group.

[a] values are significantly different from control group; ns-non significant; *p < 0.05; **p < 0.01; ***p < 0.001.

[b]values are significantly different from DEN- induced group; ns-non significant; *p<0.05;**p<0.01;***p<0.001.

Figure. 14 VITAMIN- E

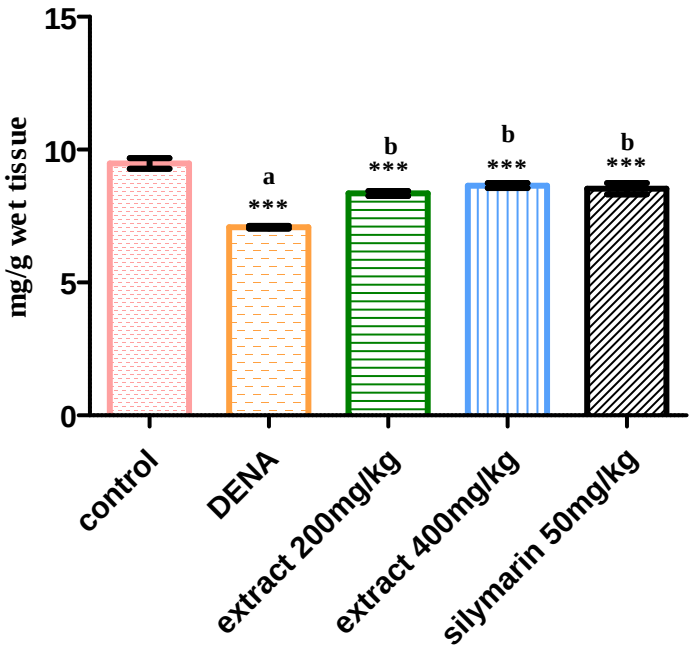


Figure. 15 VITAMIN- C

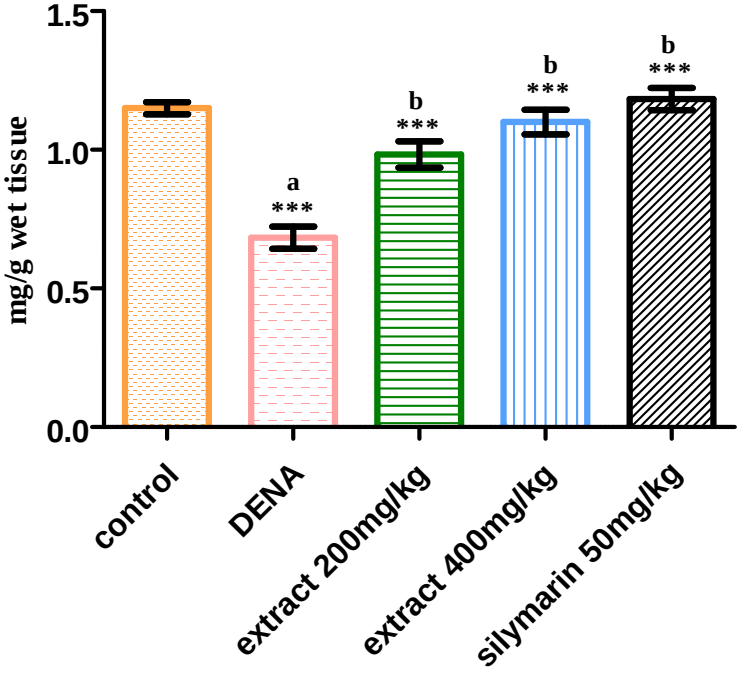
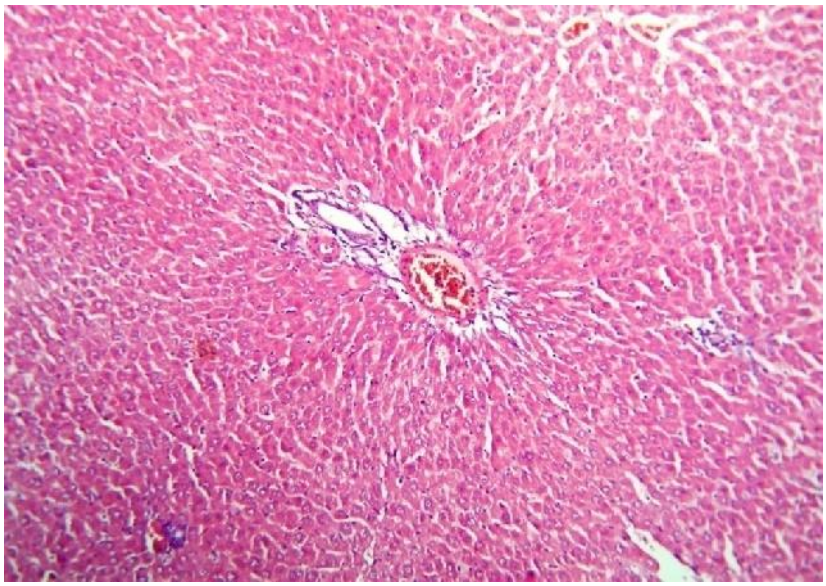
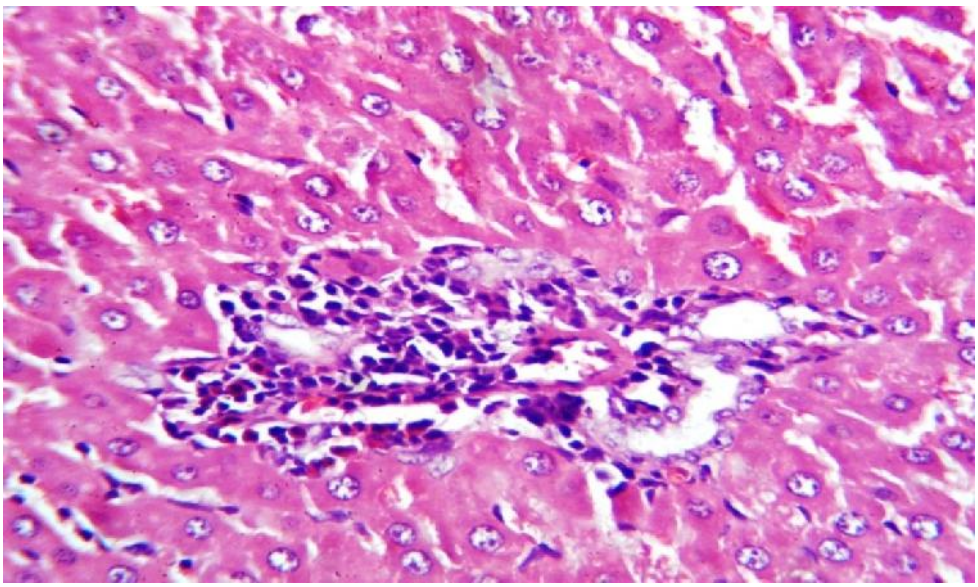


Figure. 16 Normal liver H&E 100X

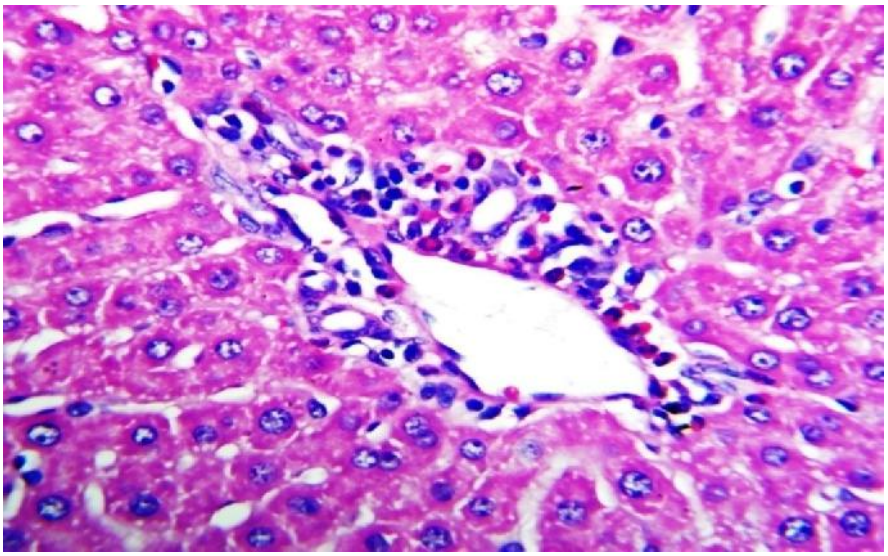


(100×) H and E stained section of liver from a normal group rat. The portal tracts, the central veins, hepatocytes, hepatic sinusoids and kuppfer cells appear normal.

Figure. 17 A Untreated H&E 400X

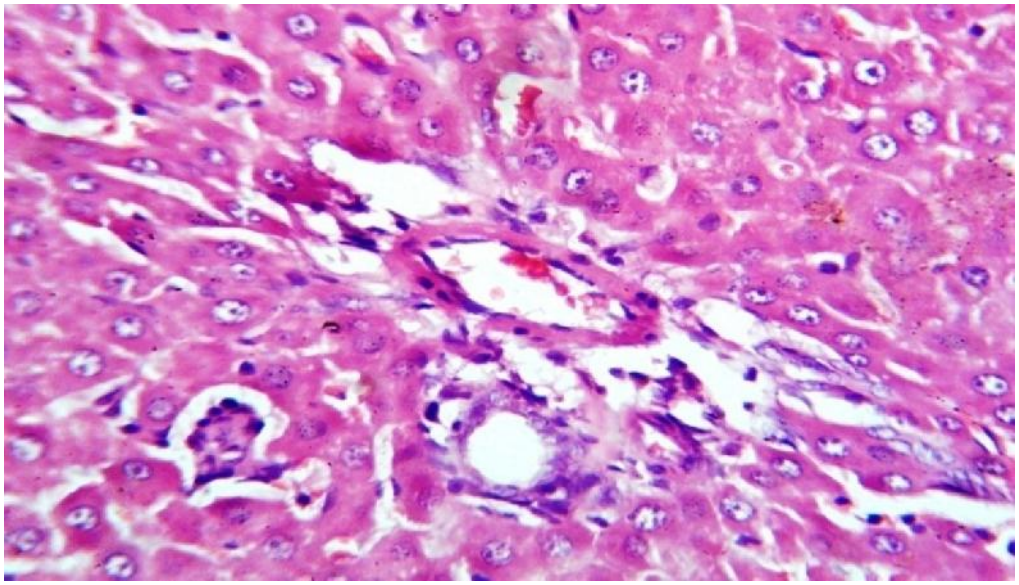


B Untreated H&E 400X

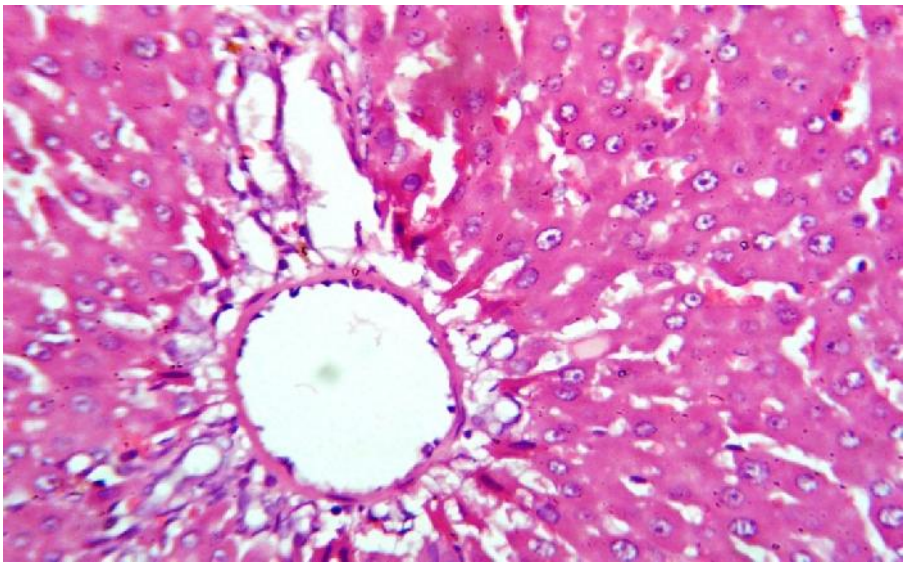


(400×) H and E stained section of liver from DEN (200mg/kg) group rat showing occasional areas of periportal inflammation. The central veins, hepatic sinusoids and hepatocytes appear normal. There is no evidence of neoplastic transformation of the hepatocytes.

Figure. 18 A Untreated H&E 400X Extract 200mg/kg H&E 400X

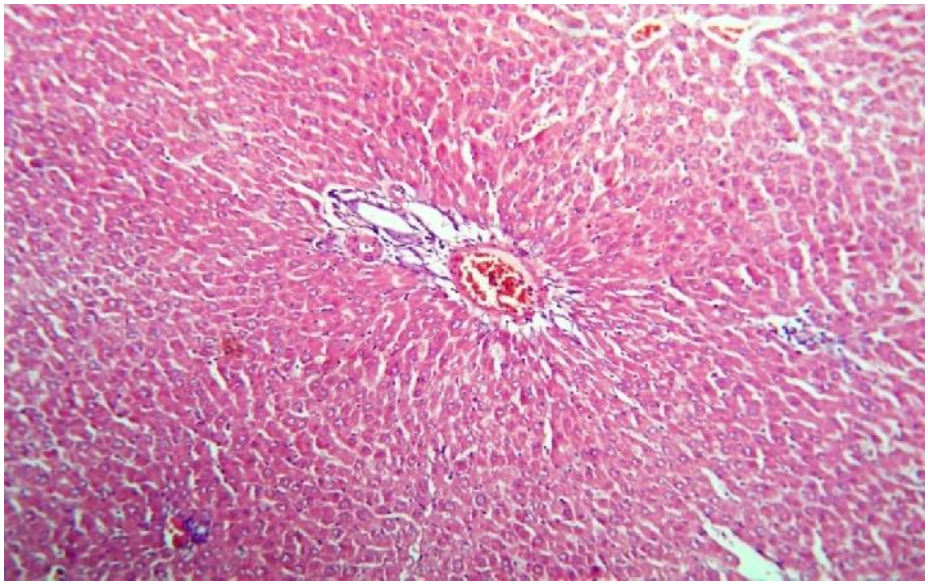


B



(400×) H and E stained section of liver from a DEN + *Couroupita guianensis* flower extract (200 mg/kg) treated group III rat showing normal architecture. The portal tracts, central veins, hepatocytes and hepatic sinusoids appear normal.

Figure. 19 A Untreated H&E 400X Extract 400mg/kg H&E 100X



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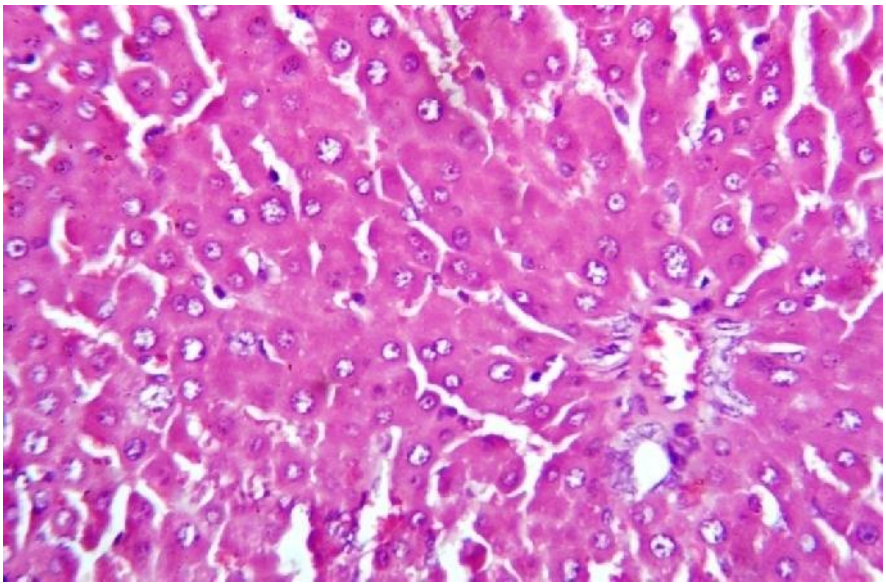
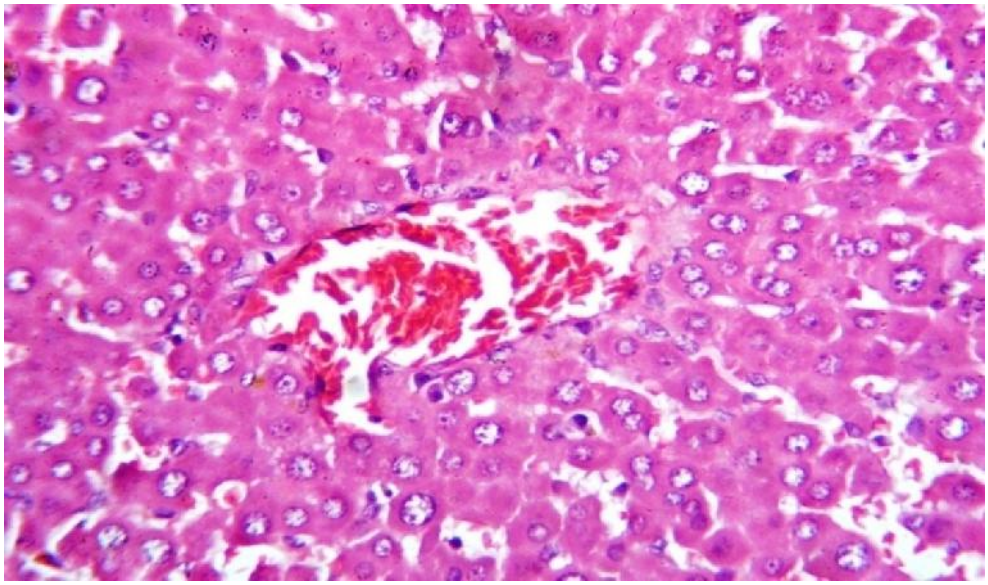
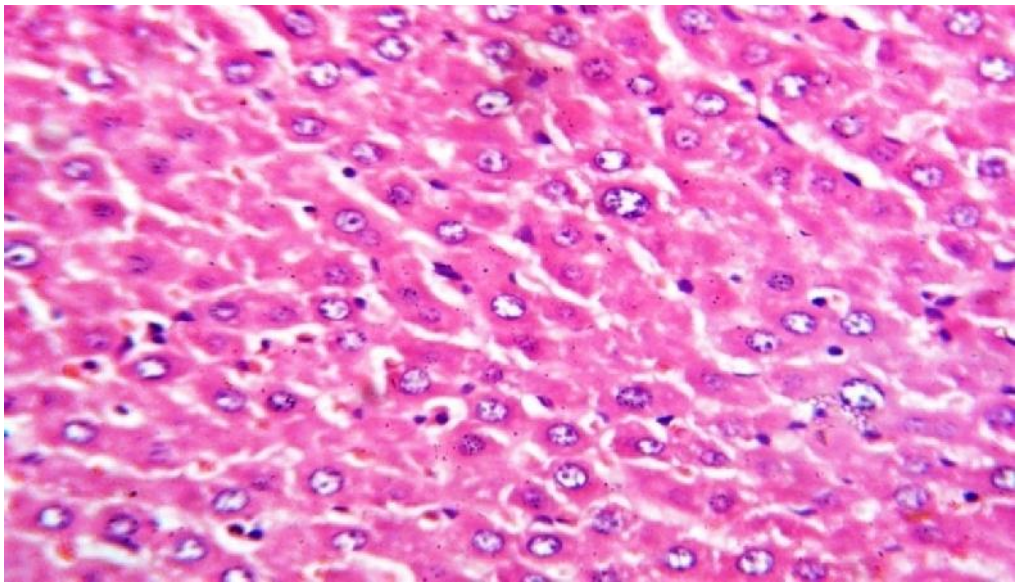


(100×) H and E stained section of liver from a DEN + *Couroupita guianensis* flower extract (400 mg/kg) treated group IV rat showing showing normal liver architecture. The portal tracts, central veins, hepatocytes and hepatic sinusoids appear normal.

Figure. 20 A Untreated H&E 400X Standard drug H&E 400X



B



(400×) H and E stained section of liver from a DEN + Silymarin (50 mg/kg) treated group V rat showing congestion in central veins. Neoplastic transformation not observed with no evidence of periportal inflammation. Hepatocytes and sinusoids appear normal.

The percentage yield of extract obtained from extraction of *couroupita guianensis* flowers using petroleum ether as solvent was found to be 2.97% w/w and 7.14% w/w with ethanol

The phytochemical examination of ethanol extract of *couroupita guianensis* revealed the presence of alkaloids, flavonoids, phenolics and tannins, carbohydrates, proteins, amino acids and glycosides.

Acute oral toxicity study was carried out as per OECD guideline 425. From the results it was observed that, the *couroupita guianensis* flowers extract is safe upto a dose level of 200mg/kg. there was no mortality and the experimental animals

did not show any toxic effect throughout the observation period of 14 days. The observation of the toxicity study is shown in table 2

The effect of *couroupita guianensis* flowers extract on α- feto protein and carcino embryonic antigen level in DEN induced liver damage is show in table 3. A significant increase ($P<0.001$) in α- feto protein and carcino embryogen antigen level is noted in diethylnitrosamine induced hepatic damage. The group of rats treated with couroupita guianensis flowers extract 200 mg/kg showed 22.06% decrease in α- feto protein and 28.34% decrease in CEA compared to untreated animals. Whereas the animals treated with 400mg/kg extract showed 40.71% decrease in α- feto protein and 39.68% decrease in CEA compared to untreated animals. The standard drug silymarin showed 51.37% decrease in α- feto protein and 46.71% decrease in CEA level.

The effect of *couroupita guianensis* flowers extract on the activities of marker enzymes in serum of control and experimental groups is shown in table 5.

A significant increase in SGOT, SGPT, ALP & total bilirubin levels were observed in rats received diethylnitrosamine. The animals treated with *couroupita guianensis* flowers extract 400mg/kg showed a significant decrease in SGOT($P<0.001$), SGPT($P<0.01$), and ALP ($P<0.001$) level with a significant decrease in total bilirubin level($P<0.001$). The total protein level which was decreased ($P<0.001$) on diethylnitrosamine showed a significant increase ($P<0.001$) on treated with extract. The standard drug silymarin restored the activities of liver function which was disturbed by diethylnitrosamine.

The effect of *couroupita guianensis* flowers extract on liver oxidant, antioxidant status of control and experimental group rats is shown in table 6. A significant increase in lipid peroxidation ($P<0.001$), with a significant decrease ($P<0.001$) in antioxidant enzyme were observed in diethylnitrosamine induced damage in liver. The animals treated with couroupita guianensis flowers extract showed a significant decrease ($P<0.001$) in lipid peroxidation with an significant increase ($P<0.001$) in antioxidant enzyme in liver. The standard drug silymarin effectively restored the oxidant-antioxidant balance, which was disturbed by diethylnitrosamine.

A significant decrease ($P<0.001$) in vitamin-C and vitamin-E was noted in liver of diethylnitrosamine control animals. Whereas the animal treated with extract and silymarin showed a significant increase ($P<0.001$) in antioxidants vitamin C and vitamin E.

DISCUSSION

Oxidative stress is a common mechanism contributing to initiation and progression of hepatic damage in a variety of liver disorders. Oxidative stress is associated with damage to a wide range of macromolecular species including lipids, proteins, and nucleic acids thereby producing major interrelated derangements of cellular metabolism including peroxidation of lipids (**Sun, 1990**). Reactive oxygen species (ROS) formed from endogenous (or) exogenous sources are highly reactive, toxic and mutagenic (**Halliwell, 1994**). Diethylnitrosamine, one of the most important environmental carcinogen, has been suggested to cause the generation of reactive oxygen species(ROS) resulting in oxidative stress and cellular injury (**Bartsch et al., 1989**). As liver is the main site for diethylnitrosamine metabolism, the production of ROS in liver may be responsible for its carcinogenic effects (**Bansal et al., 2005**) . The involvement of oxidative stress in diethylnitrosamine induced hepatotoxicity and carcinogenicity underscores the need for development of novel compound with potent antioxidant activity. In this study, diethylnitrosamine administration to rats leads to a marked elevation in levels of serum SGOT, SGPT, ALP and total bilirubin levels, which is indicative of hepatocellular damage. The increase in SGOT, SGPT and ALP levels might be due to the possible release from cytoplasm, into the blood circulation rapidly after rupture of plasma membrane and cellular damage. High levels of SGOT indicates liver damage, such as that caused by viral hepatitis as well as cardiac infraction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is release in similar manner. Therefore SGPT is more specific to the liver and is thus a better parameter for detecting liver injury (**Rao and Mishra, 1997**). These enzymes are the most sensitive markers employed in the diagnosis of hepatic damage because they are in cytoplasmic location and hence released into the circulation after cellular damage (**Wroblewski, 1959; Sallie et al., 1991**). Treatment with C*ouroupita guianensis* flower extract significantly reduced the activities of the enzymes in DEN treated rats. This indicates that couroupita guianensis flower extract tends to prevent liver damage by maintaining integrity of the plasma memberane, thereby suppressing the leakage of enzyme through membranes, exhibiting hepatoprotective activity.

Elevated ALP level may indicate cholestasis (partial or full blockade of bile ducts). Since bile ducts bring bile from the liver to gall bladder and intestine, inflammation/damage in liver cause spillage of ALP in blood stream. ALP levels typically rise to several times the normal level following the bile obstruction or intra hepatic cholestasis. Causes of elevated ALP also include bilary cirrhosis, fatty liver and liver tumor (**Quinn and Johnston, 1997**). Significant reduction in ALP levels in *Couroupita guianensis* flower extract treated group indicates the protective effect of C*ouroupita guianensis* flower against diethylnitrosamine induced hepatic injury.

Bilirubin is a yellow pigment produced when heme is catabolised. Hepatocytes render bilirubin water soluble and therefore easily excretable by conjugating it with glucuronic acid prior to secreting it into bile by active transport. Hyperbilirubinemia may result from the production of more bilirubin than the liver can process or obstruction of excretory ducts of the liver (**Tolulope Olalepe et al., 2010**). Serum bilirubin is considered as one of the true test of liver function since it reflects the ability of the liver to take up and process bilirubin into bile. Elevated levels may indicate several illness. High levels of total bilirubin in diethylnitrosamine induced stress may be due to its toxic effect. The significant reduction in the level of total bilirubin in the serum of C*ouroupita guianensis* flower extract treated rats suggest the hepatoprotective potential of *Couroupita guianensis* flowers.

Elevated of serum alpha fetoprotein (ALP) levels has been reported in several diseases including hepatocellular carcinoma. AFP is a serum protein similar in size, structure and aminoacid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatotoxic agents. It is a $72KD_a$ $\alpha_1$globulin with an uncertain biological function, is synthesized during embryogenic life by foetal yolk sac, liver and intestinal tract. AFP has high specificity for hepatocarcinoma (**Abelev, 1971; Liu et al., 2006**). Its serum concentration can be used to confirm hepatocarcinoma and for the diagnosis of tumor response to therapy. More than 90% of patients with hepatic cancer have increased serum AFP levels (**Jahan et al., 2011**). A level above 500ng/ml of AFP in human adults can be

indicative of hepatocellular carcinoma, germ cell tumors, and metastatic cancers of liver.

Carcinoembryonic antigen (CEA) is a type of protein molecule found in many different cells of the body, but typically associated with certain tumors and the developing fetus. The most frequent cancer which cause an increase in CEA is cancer of colon and rectum. Other include cancers of pancreas, stomach, breast, lung, and certain type of thyroid and ovarian conditions. Benign conditions which can elevate CEA are smoking, infections, inflammatory bowel disease, pancreatitis, cirrhosis of liver. Levels of CEA increase with an increase in tumour size. Serum values of more that 5ng/ml indicate metastasis (**Jahan et al., 2011**).

In the present study a significant increase in levels of AFP and CEA was observed in diethylnitrosamine induced hepatic injury. Treatment with C*ouroupita guianensis* flower extract showed a significant decrease in AFP and CEA levels which indicates a positive prognosis. The decrease level on *Couroupita guianensis* flower treatment prevents the neoplastic growth and reduces hepatic disorder

Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O_2., H_2O_2 and OH.) generated exceeds the antioxidant capability of the cells (**Sies, 1991**). The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress (**Khan, 2006**). Free radical scavenging enzymes like SOD and catalase protect the biological system from oxidative stress. The SOD dismutates superoxide radicals (O_2.$^-$) into hydrogen peroxide (H $_2O_2$) and O_2 (**Fridovich, 1986**). Catalase further detoxifies H_2O_2 into H_2O and O_2 (**Murray et al., 2003**). Thus SOD, catalase and glutathione peroxidase act mutually and constitute the enzymatic antioxidative defense mechanism against reactive oxygen species (**Bhattacharjee and Sil, 2006**). The decrease in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating free radicals generated during the metabolism of diethylnitrosamine. This is further substantiated by an elevation in the levels of lipid peroxidation. Lipid peroxidation (LPO) has been postulated as being the destructive process in liver injury due to diethylnitrosamine. The excessive ROS generated during diethylnitrosamine

metabolism rapidly reacts with lipid membrane. This initiates lipid peroxidation chain reaction, which leads to formation of several toxic by products such as malondialdehyde and 4-hydroxynonenal which can attach cellular targets including DNA, inducing mutagenicity and carcinogencity (**Zwart et al., 1999**). Administration of diethylnitrosamine has been reported to generate lipid peroxidation in general (**Hietnen, 1987**). The results of the present study are in accordance with these reports. Restoration in the levels of lipid peroxidation by C*ouroupita guianensis* flower extract could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids.

Excessive liver damage and oxidative stress caused by diethylnitrosamine, depleted the levels of non-enzymatic antioxidants like vitamin C and vitamin E. Non-enzymatic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system. GSH acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation (**Chaudiere, 1994**). Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and GSH (**George,2003**). It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of free radicals (**Das, 1994**). The decreased levels of these antioxidant vitamins observed during diethylnitrosamine administration might be due to the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of diethylnitrosamine. Extract treated animals showed a significant increase in vitamin C and vitamin E levels. This shows *Couroupita guianensis* flower extract maintains the level of antioxidant vitamins, thereby protecting the cells from further oxidative stress. A significant decrease in activities of GST and GR was observed in diethylnitrosamine treated rats, which may be due to decreased expression of antioxidants during hepatocellular damage. The observations are in accordance with the reports of kweon et al 2003 who demonstrated that diethylnitrosamine induced hepatocellular injury was escorted by a substantial fall in glutathione peroxidase and GST activity, which improved on administration of antioxidants. In the present study, treatment with *Couroupita guianensis* flower extract maintains the activity of GR and GST in liver. This is indicative of the potent antioxidant activity possessed by *Couroupita guianensis* flower. From the results of invivo antioxidant study, it was observed that

C*ouroupita guianensis* flower is effective in scavenging the free radicals released during the metabolism of diethylnitrosamine.

Chapter VII

Conclusion

CONCLUSION

In conclusion, the present investigation shows that the *Couroupita guianensis* flower extract exhibits excellent hepatoprotective property by restoring the hepatic marker enzymes and by stabilizing and increasing all the components of antioxidant defense system during diethylnitrosamine induced oxidative stress in rats. I suggest that the natural antioxidants and scavenging agents in *Couroupita guianensis* flower extract might be effective as hepatoprotectors and thus, may have some obvious therapeutic implications. Therefore, it seems logical to infer that *Couroupita guianensis* flowers, because of its antioxidant property, might be capable of protecting the hepatic tissue from diethylnitrosamine-induced injury.

The ethanol extract of *Couroupita guianensis* flower extract is found to be rich in flavonoids, phenolics and tannins in our preliminary photochemical screening. Since flavonoids, phenolics and tannins are powerful antioxidants, their presence in *Couroupita guianensis* flower extract flower extract might be responsible for antioxidant property, which might be involved in the hepatoprotective activity.

Chapter VIII

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